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HISTOPATHOLOGY AND HEMATOLOGY OF EXPERIMENTAL YELLOW FEVER.*

BY HENRY R. MULLER, M.D.

The guinea pigs used in the experiments to be reported here weighed about 200 to 300 grams, experience having shown that larger animals are only slightly or not at all susceptible to experimental yellow fever. Likewise in the case of dogs, it was necessary to use puppies 6 to 8 weeks old.

In order that the material obtained from animals might be comparable with *post mortem* material from cases of human yellow fever, the disease was allowed to run its natural course to death, whenever practicable, or the animals were killed while moribund. Care was taken, also, to avoid infecting the animals in an unbroken series of passages, the infection being induced by cultures of *Leptospira icteroides*, so far as possible. Noguchi has pointed out[1] that:

Guinea pigs and dogs in an unbroken series of passages become less jaundiced, the liver is less fatty, the kidneys and other tissues are more hemorrhagic, and finally death occurs with a veritable leptospiremia. The picture in such animals is quite unlike that in animals inoculated with a strain freshly isolated from human cases, or one passed through a few generations on the leptospira medium after passage through animals. Under natural conditions *Leptospira icteroides* never passes directly from man to man, but only, so far as we know, through the medium of the mosquito, *Ædes ægypti*, in the body of which it remains for many days as though in culture; hence its characteristic pathogenicity is maintained. If yellow fever were transmitted directly from man to man in an unbroken series of passages, the clinical picture might be a different one.

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* The gross and histological changes described in this article are illustrated in the colored plates incorporated in Monograph No. 20 of the Rockefeller Institute for Medical Research, published in August, 1924. The reader is referred also to the reports of Dr. Octavio Torres. *Sciencia Medica*, 1924, vol. II, pp. 219, 313, 394.

[1] NOGUCHI, H., "Researches on Yellow Fever. Comparative Etiological, Pathological, and Epidemiological Considerations: Prevention and Treatment." *The Lancet*, 1922, ccii, 1185.

Failure to avoid a long series of passages and to allow the infection to run a natural course may partly explain why Wanstrom[1] did not find marked fatty degeneration of liver and kidney. W. H. Hoffmann,[2] who also obtained unusual results, used guinea pigs which were in the 20th to the 50th passages of a long series of infections with a strain passed from animal to animal.

Histopathological Changes Induced in Guinea Pigs by the Experimental Infection.

Liver.—In general, it has been found that in guinea pigs inoculated with *Leptospira icteroides*, in which the infection has been allowed to run a natural fatal course, the liver is paler than normal and looks in the gross like a fatty liver. Microscopically, the normal structure is seen to to be greatly disturbed, the trabeculae being distorted, and the sinuses compressed. The liver-cells are slightly separated from one another, so that the cells present a loose texture. The cells are swollen and granular and have numerous fine, round, and irregular vacuoles. Irregularly scattered throughout the section are numerous small groups of cells, with indistinct outlines and with nuclei in various stages of karyolysis or karyorrhexis, or entirely without nuclei. Associated with these necrobiotic changes, numerous mitotic figures are constantly found. All phases of normal cell-division are encountered, showing that active regeneration of liver-cells is taking place, following the death of cells.

Fatty infiltration of the liver of these guinea pigs is a very constant finding, as is brought out by Scarlet R or Sudan III. It may be very severe, involving almost the entire lobule; or it may be less marked, and limited to the periphery of the lobule; or it may be scattered diffusely throughout the lobule in small foci.

Small foci of hemorrhages may be present. Polynuclear leucocytes are sometimes encountered among the swollen liver-cells, apparently

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[1] WANSTROM, RUTH C., "Occurrence and Staining of *Leptospira icteroides* in Guinea Pigs Inoculated Experimentally, with a Study of the Lesions Produced." *Journ. Infect. Dis.*, 1924, xxxiv, 110.

[2] HOFFMANN, W. H., "The Histopathology and Haematology of Guinea Pigs Infected with *Leptospira icteroides.*" *Journ. Trop. Med. and Hyg.*, 1922, xxv, 353.

lying in the compressed sinuses, and there is often a round-cell infiltration of the periportal connective tissue.

Kidneys.—In most cases the epithelium is greatly swollen, so that the lumen of the convoluted tubules is either completely obliterated or filled with pink-staining granular material. Hyalin and granular casts are frequent. The cytoplasm is granular and finely vacuolated. Frequently the degenerative processes in the cells have gone on to complete necrosis, so that individual cells or most of the cells of a tubule are without nuclei or have nuclei in various stages of karyorrhexis or karyolysis. In a large percentage of instances, mitotic figures are present, showing that repair of the damage is taking place. Mitotic figures are generally not found in tissues of animals dying before the 5th day of disease.

Fatty infiltration, usually not quite so severe, nor quite so constantly present as in the liver, is found in the cells of either the convoluted or the collecting tubules, or in both. Minute hemorrhages in the cortex and medulla, or hemorrhages into lumen of tubules, or larger hemorrhages beneath the capsules, may be present. Congestion of the glomeruli may also appear. Cellular infiltration is rare in the interstitial tissue and is never found in the glomeruli.

Heart.— Petechial hemorrhages into the papillary muscles and subendocardial and subepicardial are frequently observed. Zenker's degeneration was never seen, notwithstanding careful search for it.

Fatty infiltration usually occurs in patches; it is present in a large number of instances, but not so constantly as in either the liver or the kidney.

Lungs.— Corresponding with the hemorrhagic spots seen in the gross, the sections show foci in which hemorrhage has occurred into a number of adjacent alveoli.

Gastro-Intestinal Tract.—In the mucosa of the stomach are hemorrhages, sometimes quite extensive, corresponding with those that are uniformly seen in the gross. Hemorrhages often occur in the mucosa of the colon.

Spleen.—As would be expected from the practically unchanged appearance of the spleen in the gross, the microscopic sections present only rarely very slight changes. In some cases there may be a moderate or very slight diffuse polynuclear infiltration. Occasionally the

sinuses are slightly dilated. Only in a few instances is there a pha-
gocytosis of red-cells. The lymph follicles are not changed. Pigment
is rarely found.

Lymph Nodes.—The lymph nodes, such as those of the mesentery,
very rarely are enlarged, and the lymph follicles and Peyer's patches
of the intestines are never involved in cases which are free from sec-
ondary infection, such as paratyphoid.

The lesions found in guinea pigs[1] are illustrated by the following
protocols of animals inoculated with the Brazilian strains of *Leptospira
icteroides.*

Guinea Pig XXII–46.—Inoculated March 22, 1924, with 0.5 cc. of
culture of Brazilian Strain 3. March 24, temp. 104.8°F. (40.5°C.).
March 25, temp. 105.2°F. (40.7°C.). March 26, temp. 103.8°F.
(39.9°C.); blood obtained by heart puncture showed one leptospira
after 5 minutes' search with the dark-field microscope. March 27,
temp. 104.6°F. (40.7°C.). March 28, temp. 103.6°F. (39.8°C.).
March 29, animal markedly jaundiced; temp. 101°F. (38°C.); mori-
bund; killed.

Autopsy.—Marked general jaundice; hemorrhages of skin and
subcutaneous tissues. Stomach contained black and blood-stained
particles; hemorrhages in the mucosa of the stomach, in the lungs, and
a few in the capsules of the kidneys. One leptospira found by dark-
field examination in kidney suspension, but none in either citrated
heart-blood or liver suspension. Cultures of spleen in broth and on
plain agar remained sterile.

Liver.—Parenchyma cells slightly separated from one another,
and are swollen so that the sinuses are almost all obliterated. Cyto-
plasm granular, pink-staining, and finely vacuolated. Some nuclei
are fragmented; no mitotic figures found. Around the structures of
the portal canals is an infiltration of large mononuclears, with a few
lymphocytes and polynuclears. A few polymorphonuclears and
lymphocytes are scattered among the liver-cells. Staining with Scarlet
R reveals numerous fine fat-droplets in the liver-cells occupying the
central half of the lobule. Levaditi sections fail to show the leptospira.

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[1] The tissues in all cases were fixed in formalin and stained with hematoxylin
and eosin; when fat was to be demonstrated, they were stained with Scarlet R or
Sudan III.

Kidneys.—Swelling of the epithelium of the convoluted tubules is so marked that the lumen is obliterated. Cells granular and finely vacuolated. A few minute hemorrhages are present between the tubules and within their lumens. With Scarlet R a marked fatty infiltration of the epithelium of the collecting tubules can be demonstrated. Levaditi sections reveal numerous leptospira in the cortex of the kidney, outside the tubules, as well as within the epithelial cells.

Heart.—Minute hemorrhages are present. Around the small blood-vessels are collections of large mononuclears and a few polymorphonuclears. In Scarlet R sections, patches of muscle-fibres are seen to contain numerous fine drops of fat.

Stomach.—Extensive hemorrhages into mucosa and between muscularis mucosae and muscularis are present. There are patches of necrotic mucosa, sharply demarcated from the rest, and surounded by a zone of polynuclears at the base and extremities. The surface of the necrotic patch contains brown blood-pigment.

Intestines.—Peyer's patches not enlarged; hemorrhages present in mucosa of large intestine.

Guinea Pig XXII–72–A.—Weight 200 grams. Inoculated April 7, 1924, with 1 cc. citrated heart-blood from infected guinea pig 68 B, which had been inoculated with blood from guinea pig 57 A; the latter had received 0.5 cc. culture of *Leptospira icteroides*, Brazilian Strain 5. April 12 and 13, temp. 104°F. (40°C.). April 14, temp. 102.2°F. (39°C.); blood obtained by heart-puncture contained 4 leptospira in 150 fields, and cultures made with the blood showed marked growth of *Leptospira icteroides* on April 22; cultures on broth and agar remained sterile. April 15, animal died.

Autopsy.—Jaundice; hemorrhages into subcutaneous tissues; hemorrhagic spots in lungs; hemorrhages around both kidneys; one large hemorrhagic spot in stomach, near cardia; and several small spots on external wall of stomach and intestines; liver is light brown and friable; spleen is normal.

Liver.—The liver-cells, which are large and polyhedral, have a tendency to be separated from one another. The cytoplasm is granular and finely vacuolated. A few scattered cells are conspicuous because they stain more deeply red. Most of the nuclei are spherical and appear normal, but there are many undergoing mitotic division. In the

periportal connective tissue there is an infiltration of large mononuclears and a few lymphocytes and polynuclears. In Scarlet R sections, the liver-cells are found to contain fat-droplets. Nearly the entire lobule is involved, but a narrow zone on the periphery of each lobule is less affected. In Levaditi sections, the leptospiras are found in large numbers, uniformly scattered, lying chiefly between, but also within, the liver-cells.

Kidneys.—The cells of the convoluted tubules are swollen, granular, and vacuolated. The lumen in some places is nearly obliterated by the swollen epithelium. Many tubules, both convoluted and collecting, contain red-blood-cells, others pink-staining, finely-granular débris, and casts. Small hemorrhages are present in the interstitial tissue. The glomeruli are slightly congested. Scarlet R brings out small foci of fat in the epithelium of the collecting tubules. In Levaditi sections, a rather large number of leptospiras are found, chiefly between the tubules, but also within the kidney-cells.

Heart.—In the papillary muscles and beneath the endothelium are found minute hemorrhages, in the vicinity of which are polynuclears and large mononuclears. Scarlet R sections show that numerous patches of muscle-fibres contain many fine drops of fat. Levaditi sections reveal a few isolated leptospiras in the muscle-fibres.

Stomach.—Numerous and extensive hemorrhages are present in the mucosa and muscularis.

Intestines.—Peyer's patches normal. Hemorrhages present in mucosa of colon.

Spleen.—Normal.

Skeletal Muscles.—No Zenker's degeneration; a few petechial hemorrhages in abdominal muscles.

Histopathological Changes Induced in Dogs by the Experimental Infection.

The following descriptions are based on histopathological study of tissues of 2 puppies, of a litter about 8 weeks old, which were inoculated with a rich culture of *Leptospira icteroides*, Brazilian Strain 5, each receiving 5 cc., one subcutaneously and the other intraperitoneally. On the 7th day after inoculation, both dogs died, and autopsy revealed deep jaundice; "coffee-ground" material in the stomach; pale, soft

livers; pale kidneys in one, and hemorrhagic kidneys in the other animal. Both had very few hemorrhages in the skeletal muscles. In one instance, there was no urine in the bladder; but the bladder of the other animal contained 2 or 3 drops of highly albuminous urine, as shown by Heller's test.

Liver.—In young dogs the changes in the liver are more striking than in guinea pigs. There is evident very severe disturbance, so that the trabeculae can scarcely be distinguished. The trabecular arrangement of cells is lost; the individual cells are swollen, granular, vacuolated, and slightly separated from one another. There are minute foci of cells without nuclei, or with nuclei which are pale and indistinct. Great variations appear in the staining quality of the nuclei. Mitotic figures, which point to an extensive reparative process in the liver, are frequently encountered. In the compressed sinuses are many polynuclears. Congestion and hemorrhage are present, and in one puppy the liver is the seat of large, round-cells stuffed with erythrocytes. The liver of one dog, as shown by staining with Scarlet R, presents fatty infiltration of the entire lobule; in the other animal the fat is in the central and intermediate zones. There are also droplets of fat in the epithelium of the bile-ducts.

Kidneys.—In the kidneys of one animal, there are complete necrosis of the epithelium and extensive hemorrhages into the tubules, interstitial tissue, and glomeruli. In the other animal, the cells of the convoluted tubules are swollen and granular, and the lumen of the tubules is occluded by swollen cells. Many cells are without nuclei (indicating death of cells) and in many others mitotic figures are present, pointing to a reparative process. The urinary bladder is normal in both animals.

The kidneys in both instances have marked fatty infiltration of the collecting tubules, as is brought out with·Scarlet R. In addition, the kidneys showing extensive hemorrhages have also fatty infiltration of the endothelium of the glomeruli.

Heart.—Both puppies show (1) an infiltration of fat in the muscle-fibres, in the form of fine droplets, and (2) a few petechial hemorrhages. Zenker's degeneration absent.

Lungs.—There are a very few small foci of hemorrhages into the alveoli. They are very much fewer in number than those found in guinea pigs.

Spleen.—In one animal the spleen is practically normal; in the other, it is slightly congested and has a few phagocytes filled with red-blood-cells.

Mesenteric Lymph Nodes.—A moderate number of erythrophago-cytes are present.

Pancreas.—There is a very marked interstitial infiltration of poly-nuclears in both animals. A few minute hemorrhages exist in the interstitial tissue of one animal.

Stomach.—Hemorrhages, rather extensive, are found in the sub-mucosa, and congested vessels and small hemorrhages are present in the mucosa.

Intestines.—Peyer's patches uninvolved.

Skeletal Muscles.—The abdominal muscles are the seat of minute hemorrhages, but no degeneration of the muscle-fibres is present. The hemorrhages in the skeletal muscles are very much less numerous and extensive than in guinea pigs.

Levaditi Sections.—The leptospira could be demonstrated in varying numbers in practically all the organs—liver, kidneys, heart, lung, spleen, intestinal wall, pancreas, lymph nodes, and skeletal muscles. Many of the organisms were fragmented forms.

Histopathological Changes in Experimentally Infected Monkeys.

The tissues examined were obtained from one of 2 animals of the species *Cebus macrocephalus* which had been inoculated with mixtures of cultures of Brazilian Strains 3 and 5. The first rise of temperature occurred 60 hours after inoculation. The animal died on the 7th day after onset of fever.

Liver.—In the hematoxylin and eosin sections the liver trabeculae are distinct, and the liver-cells are large, polyhedral, and vacuolated. The vacuoles are variable in size, and there are 6 to 10 in each cell. The closely packed vacuoles make the cytoplasm appear reticulated. The nuclei are large and round, and most of them lie towards the centre of the cells. The blood-sinuses are dilated, and filled with blood. The blood-vessels and bile-ducts of the portal canals are normal.

In Scarlet R sections, the fat globules fill almost the entire liver-cell, and every lobule, except for a narrow zone around the central vein, is completely fatty. Towards the periphery of the lobule, the fat-glo-

bules tend to be larger and conglomerate. The epithelium of the bile-ducts shows no fatty change.

Kidneys.—In the hematoxylin and eosin sections, the cells of the convoluted tubules have indistinct outlines and appear as pink-staining, granular, and vacuolated masses almost completely occluding the lumen. The nuclei are smaller than normal, and pyknotic. Minute hemorrhages are present in the medulla of the kidney. The glomeruli are normal.

In Scarlet R sections, the cells of the convoluted and collecting tubules contain numerous closely packed fine fat-droplets, situated mainly at the base of the cell. This fatty infiltration is very marked.

Heart.—There are no hemorrhages nor any form of degeneration, such as Zenker's. In Scarlet R sections, numerous fine fat-droplets are uniformly sprinkled throughout the entire length of all the muscle-fibres.

Lungs.—The alveoli are free from exudate, but there is a slight degree of chronic, productive, inflammatory tissue around the bronchioles.

Stomach.—The mucosa presents extensive *post mortem* change. There are defects in the mucosa, which extend part or all of the way to the *muscularis mucosae*, and these are filled with dark-brown, coarse and fine granules of blood-pigment. The cells lining the edges of the defects contain pigment in the cytoplasm. By Perles' Prussian-blue method for demonstrating hemosiderin (derived from the hemoglobin of red-blood-corpuscles) the pigment stains blue, where it is in contact with the epithelium, and the mucosal defects also have blue linings. The presence of blood in the "coffee-ground" material in the stomach is thus evident.

Intestines.—Peyer's patches uninvolved. Mucosa normal.

Spleen, adrenal, pancreas, and *large intestine* are all negative.

Levaditi sections fail to reveal leptospira in any of the organs; in view of the fact, however, that leptospira can rarely be demonstrated in human autopsy material, this negative finding is not remarkable.

Hematological Studies of Experimentally Infected Guinea Pigs.

As preliminary to a study of the blood-changes in guinea pigs experimentally infected with yellow fever, a record was kept of the

TABLE 1.

Blood Counts on Guinea Pigs Fatally Infected with L. icteroides.

	Guinea pig XX-92A 0.01 cc. virus (arias + vilela)		Guinea pig XX-92B 0.01 cc. virus (arias + vilela)		Guinea pig XX-93B 0.1 cc. virus (arias + vilela)		Guinea pig XX-94A 1.0 cc virus (arias + vilela)		Guinea pig XX-94B 1.0 cc. virus (arias + vilela)	
	Temp. and icterus	Blood counts	Temp. and icterus	Blood counts	Temp. and icterus	Blood counts	Temp. and icterus	Blood counts	Temp. and icterus	Blood counts
Before inoculation		9,260 / 4,560,000		7,700 / 5,820,000		5,850 / 4,590,000		7,500 / 6,010,000		7,950 / 4,620,000
1st day after inoculation										
2nd " " "		12,200		8,050		6,400 / 5,350,000		6,750		6,900
3rd " " "										
4th " " "	105.6°	9,640	104.4°	9,280	106°	3,150	104.4°	5,600	103°	8,000
5th " " "	104.4°		.105.4°		105.2°		102.4°		104.4°	
6th " " "	101.8°	8,700 / 4,800,000	102.8°	5,400 / 5,270,000	101.9°	3,520 / 4,440,000	102°	5,300 / 4,720,000	101.6°	5,000 / 4,680,000
7th " " "	104.6°		100.2° ++		103° ++	70% Tall.	102.4° ++++	9,400 / 3,340,000 60% Tall.	102° ++++	

8th day after inoculation	102.6° ++	102.6° +++	100.6° ++	Died Autopsy, typical	99° ++++
9th " "	Died Autopsy, typical	Died Autopsy, typical	98° +++	No blood obtainable from ear for count	Died Autopsy, typical
10th " "			95° ++++		
11th " "			Died Autopsy, typical		

blood-counts and hemoglobin estimates of a large number of normal guinea pigs (250 to 300 grams) such as are used for transmission experiments in yellow fever. The blood was obtained by puncturing the ear veins.

The average number of leucocytes in 60 normal animals was 8,365 per cubic millimeter. Of the 60 animals, 13 had total leucocyte counts of over 10,000, and 9 had counts under 5,000. The highest count on any one animal was 23,500, and the lowest 4,120 per c.mm. There appear, therefore, to be wide variations in the normal white-counts and the unusually high counts (above 10,000) seemed to be peculiar to certain apparently quite normal animals. It was interesting to find that, in some instances, animals of the same litter all had high normal counts,—as, for example, the following in a litter of four: 14,400; 16,700; 23,500; 12,800.

The average total red-blood-cell count in 50 normal guinea pigs was found to be 5,480,440 per c.mm. The highest individual count was 6,930,000, and the lowest 4,340,000.

The hemoglobin estimates were made by the Tallquist method. The average percentage in 33 normal animals was 80. (Later it was found that much higher readings are obtained by the Sahli method— between 100 and 110%).

Guinea pigs which were inoculated with virulent cultures of *Leptospira icteroides*, and of which blood-counts were made, may be divided into 3 groups: (1) those which had fatal infections; (2) those which had infections, but which were killed before the natural termination of the disease; and (3) those which passed through infections and recovered.

As indicated in Table 1, 5 guinea pigs were inoculated with a mixture of cultures of *Leptospira icteroides* (Guayaquil and Peruvian strains) with citrated blood from a guinea pig infected with the Guayaquil strain of *L. icteroides*. The course of the infection was typical in each instance, and 3 of the animals died on the 8th, 9th, and 11th days, respectively, after inoculation. The autopsy findings were typical and uncomplicated.

On the 2nd day after inoculation, 2 of the 5 animals showed a slight drop in the leucocyte-count, from 7,500 to 6,750, and from 7,950 to 6,900, respectively. Of the other 3, 2 had only slight rises in total

counts on the second day after inoculation, so slight that they may be disregarded (from 7,700 to 8,050, and from 5,850 to 6,400, respectively). In one animal only was there an increase, from 9,260 to 12,200. During the course of fever the leucocyte-counts gradually fell in each instance, to slightly below normal, the largest drop being one of 40%, which occurred on the 6th day after inoculation, in the animal which died on the 11th day of the disease. In this pig, no blood was obtainable from the ear on the 9th day; hence no further counts were made.

In one instance, that of the animal which died on the 8th day, a count made on the day before death showed a rise to 9,400 from the animal's normal count of 7,500.

Red-cell-counts made on these 5 animals, on the 6th day after inoculation, showed practically no change from the normal, except in the animal which died on the 8th day, in which a reduction of red-cells to 4,720,000 and 3,340,000 took place on the 6th and 7th days, respectively. This pig gave a reading of 60% hemoglobin by the Tallquist method on the 7th day. Unfortunately, only one other hemoglobin estimate was made on this series of animals, on the 7th day in the guinea pig which died on the 11th day; the percentage was then 70%.

Of 5 guinea pigs which were typically infected with variable quantities of culture of *L. icteroides* (Guayaquil strain) and were killed between the 5th and 9th days after inoculation (Table 2), all showed a gradual reduction in the total number of leucocytes on the 1st and 2nd days after inoculation. This reduction continued, with some slight fluctuations, and the count remained well under the initial normal one except in one instance,—in which, on the 7th day after inoculation, the leucocytes rose 47% above the normal. In 4 of 5 animals the red-cells remained close to normal—or even, during the course of the fever, showed a slight numerical increase.

The hemoglobin likewise showed very slight changes from the normal.

There were 3 guinea pigs in which infection occurred following the inoculation of *L. icteroides* culture, and in which recovery took place (Table 3). Although all of these animals ran a typical course of fever lasting 3 to 5 days, but one was jaundiced, and only slightly. In one animal of this series, the number of leucocytes remained stationary

TABLE 2.

Blood Counts on Guinea Pigs Infected with L. icteroides and Which Were Killed.

	Guinea pig XX-119A 0.1 cc. arias culture		Guinea pig XX-119B 0.1 cc. arias culture		Guinea pig XX-120 A 1.0 cc. arias culture		Guinea pig XX-120B 1.0 cc. arias culture		Guinea pig XXI-117A 0.4 cc. arias culture	
	Temp. and icterus	Blood counts	Temp. and icterus	Blood counts	Temp. and icterus	Blood counts	Temp. and icterus	Blood counts	Temp. and icterus	Blood counts
Before inoculation		16,700 5,570,000 80% Tall.		23,500 4,820,000 70%		12,800 4,730,000 90%		20,000 4,500,000 90%		13,720
1st day after inoculation	101.6°	15,000 80% Tall.	100.6°	19,100 75%	101.2°	9,680 5,066,000 90%	102°	14,000 5,000,000 90%	104.2°	11,565
2nd " "	101.9°	13,200 5,160,000 90%	101.8°	15,000 4,520,000 80%	101.4°	8,900 4,900,000 80%	101.2°	11,050 5,000,000 85%	104°	
3rd " "	103.4°		103.8°		102°		103.4°			8,200
4th " "	102.4°	13,900 4,900,000 75%	105.4°	23,030 5,520,000 80%	104.8°	13,000 5,500,000 80%	104.4°	15,200 5,500,000 75%	104.6°	
5th " "	105.2°	11,000 5,300,000 80%	104° +++ Killed	19,700 3,400,000* 65%	104°	12,500 4,810,000 85%	101.2° +++	14,000	105.2°	4,365

6th day after inoculation		Autopsy, typical Leptospira in blood	103.4°		103.8° + 7,800
7th " "	102.8°		104.4° 18,800 5,200,000 85%		104° +++ 10,480 4,300,000 70%
8th " "	102° +++ Killed Autopsy, typical Leptospira in blood Levaditi sections +		103.6° ++ Killed Autopsy, typical No Leptospira in blood Levaditi sections –		101.8° +++ Killed 7,720 4,700,000 65%
9th " "				98° ++++ Killed Autopsy, typical Leptospira in blood and liver emulsions	Autopsy, typical No Leptospira in kidney or liver emulsions

* Bled from heart.

TABLE 3.

Blood Counts on Guinea Pigs Infected With L. icteroides and Which Recovered.

	Guinea pig XX-93A 0.1 cc. virus (arias + vilela)		Guinea pig XXI-116A 0.4 cc. arias culture		Guinea pig XXI-123B 1.0 cc. vilela culture	
	Temp. and icterus	Blood counts	Temp. and icterus	Blood counts	Temp. and icterus	Blood counts
Before inoculation		15,600 4,640,000		7,800		4,040
1st day after inoculation		16,000	103.4°	9,800	103°	11,960
2nd " " "		18,400 5,340,000	102.5°		101.5°	
3rd " " "				8,280	101.5°	4,940
4th " " "	106.4°	10,700	104°		104.5°	
5th " " "	104.2°		104.2°	9,200	104.6°	6,560
6th " " "	104°	7,720 4,670,000	104.4° ±	11,200	103°	8,140
7th " " "	104°	12,200 4,030,000 70% Tall.	105°	8,660 5,418,000 .	105.6°	9,920 6,560,000 75%
8th " " "	104.3°		103.2° +	9,280 4,660,000 70%	103.4°	
9th " " "	100.5°	15,660 4,670,000 50%	103.5°		102.8°	
10th " " "	100.2°		102°		101.5°	
11th " " "	102.5°		101.8°		102°	
12th " " "	101.8					
13th " " "	101°	14,600 4,940,000 80%				
14th " " "	102.8°					

during the first 24 hours after inoculation (15,600–16,000). In another the count was slightly increased after 24 hours (7,800 to 9,800), and in the 3rd there was a marked increase (from 4,040 to 11,960). Both these animals had a leucocyte-count very slightly above the normal throughout the course of fever, while in the 1st animal the leucocyte-counts remained low during the febrile period, and gradually returned to normal on the 9th day after inoculation (first afebrile day of con-valescence).

The red-cell-count in the first guinea pig remained at, or slightly above, normal throughout the period of fever, and on the 1st and 5th days of convalescence. Its hemoglobin, however, dropped from 70% (on the 4th day of fever) to 50% (on the first afebrile day). In the 2nd animal the number or red-cells dropped slightly on the first afebrile day, and its hemoglobin on that day was 70%.

SUMMARY.

In young guinea pigs, young puppies, and in a monkey (*Cebus macrocephalus*) experimentally infected with yellow fever, the his-tological changes, although varying in degree, are similar to those found in human cases.

In experimental yellow fever in guinea pigs a steady and persistent leucopenia of moderate degree, during the course of severe infections, is the rule. In occasional instances, an initial or a preagonic leucocy-tosis may be present. In extremely mild infections there may be a slight leucopenia, or a mild leucocytosis during the febrile period, according to the individual case.

The number of red cells usually remained within normal limits, although a marked reduction occasionally took place, and in some instances there was even a slight increase.

The hemoglobin was generally normal. In some animals, it was a trifle below the normal.

These findings are similar to those of yellow fever in man, as re-corded in the literature.

ACTION OF CERTAIN BIOLOGICAL, CHEMICAL, AND PHYSICAL AGENTS UPON CULTURES OF LEISH-MANIA; SOME OBSERVATIONS ON PLANT AND INSECT HERPETOMONADS.

By HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Cultural Requirements of Leishmania.

Rogers was the first to show that the non-flagellated forms of Leishmania develop into flagellates in appropriate fluid media, and in this form can be maintained in an indefinite series of subcultures. The blood agar slant of Novy and MacNeal, or the same medium without the addition of peptone and meat-infusion (Nicolle) has been widely adopted for the cultivation of Leishmania, the organisms growing in the condensation water and occasionally even on the surface, as minute dew-point colonies.

Wenyon,[1] in 1921, reported good results in the cultivation of protozoa by means of a semi-fluid agar medium to which about 1 cc. of rabbit whole blood is added to each tube at the time of use; and Kligler[2] employed a similar medium, but with the addition of 0.1 per cent of glucose, for *L. tropica.* I have found that a medium in use by me for several years for the cultivation of the leptospira group yields an excellent growth of Leishmania, 2 strains of *Leishmania brasiliensis* having been isolated by this means from 6 cases selected by Dr. Lindenberg among the patients in his Leishmaniasis ward of the Santa Casa Hospital of São Paulo.[3] One of the advantages of this medium is that the organisms, once grown, remain viable for many months without subculturing, and that the medium remains serviceable for many weeks when preserved at a low temperature (18°C. or below). Another, perhaps more important, advantage is the

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[1] Wenyon, C. M.; *Trans. Roy. Soc. Trop. Med.*, 1921, xv, 141–155.

[2] Kligler, I. J.; *Am. Journ. Med.*, 1924, iv, 69.

[3] Noguchi, H., and Lindenberg, A.; *Am. Journ. Trop. Med.*, in press.

readiness with which a large quantity of the organisms can be obtained. The growth of the various species of Leishmania on this medium becomes readily recognizable after a few days (at 18–20°C.) as a grayish-white surface haze, which continues to increase in depth until within a week a scum-like colony of 2–4 mm. in thickness covers the uppermost portion of the medium. When the rich growth is skimmed off, another mass of organisms grows in the same tube, and the process can be repeated every week or so until the medium is exhausted. The pure mass of organisms thus obtained is composed of flagellated and actively motile forms. If the first growth is not removed, the organisms remain viable for several months but ultimately undergo the usual degenerative changes.

Leishmania infantum,[4] *Leishmania tropica*,[4] and *Leishmania brasiliensis* have grown chiefly on the surface. The strain of *Leishmania donovani* in my hands[1] has shown a tendency to grow also in a zone several centimeters below the surface, where oxygen does not freely penetrate. No growth has been obtained in an atmosphere of nitrogen, hydrogen, or carbon dioxide,—hence all the Leishmania culture strains are to be regarded as obligate aerobes. The addition of NaOH to the medium beyond pH 8.2, or of HCl beyond pH 5, renders it unsuitable for the growth of these organisms.

Biological Properties of Leishmania.

Immunity.

The relation between *Leishmania tropica* and *Leishmania donovani* has been widely discussed, but no differentiation has been brought out from the laboratory standpoint. Animal experiments have seemed to indicate the identity of *Leishmania donovani* and *Leishmania infantum* (Nicolle and Laveran).[5]

Much of the unsatisfactory test-tube experimentation of the earlier days was undoubtedly due, at least in part, to the lack of means of

[4] The strains of *Leishmania donovani* and *L. infantum* were obtained through the courtesy of Prof. C. A. Kofoid, of the University of California; the strain of *L. tropica* was furnished me by Major Henry J. Nichols, of the Army Medical School.

[5] LAVERAN, A.; "Leishmanioses." Paris, 1917, page 55.

obtaining sufficient quantities of the organisms for immunity work. With cultures grown on the semi-fluid medium described, however, it has been possible to immunize rabbits with the various strains of Leishmania. Rich live cultures of a given strain were injected intravenously on 4 successive occasions at 5- to 7-day intervals. The serums of these rabbits were tested for agglutination with the homologous and heterologous strains, and the results were clear-cut and decisive. The anti-*donovani* and anti-*infantum* serums agglutinated *L. donovani* and *L. infantum* reciprocally,—that is, these 2 strains were serologically identical. On the other hand, neither of these serums showed any agglutinating property for *Leishmania tropica* or *Leishmania brasiliensis*. The anti-*tropica* serum agglutinated only the *tropica* strain and the anti-*brasiliensis* only the *brasiliensis*. The titres of these anti-serums were such that a 1:10 dilution caused a prompt and powerful agglutination, while 1:100 was moderately active against the homologous strains. The agglutination tests appear to permit the separation of at least 3 distinct species of Leishmania, (1) *L. donovani* of kala azar, and *L. infantum*, with their dominant visceral affinity, (2) *L. tropica* of oriental sore, with its essentially dermal effects, and (3) *L. brasiliensis* of the New World, with its pronounced mucodermal predilections. Cultures obtained on a medium containing 10 per cent of homologous immune serum grow in small clumps, while the growth on media containing normal or heterologous (Plate A) immune serums is smooth and scum-like. The immunological differentiation of these 3 distinct species of Leishmania may have an important bearing on the future development of serum treatment or vaccination in various forms of Leishmaniasis. Because of the limited number (2) of Brazilian strains used in the present series of experiments, the possibility of the existence of other varieties among Brazilian cases is not excluded.

Resistance to Various Agents.

As a means of obtaining information regarding the Leishmania as a living cell, various physical, chemical, and biological agents have been applied to the actively growing flagellate forms of the 4 strains, *L. tropica*, *L. brasiliensis*, *L. donovani*, and *L. infantum*.

Tonicity.—Leishmania underwent instantaneous plasmoptysis when suspended in distilled water, the flagella becoming detached and immobile, and the cell-body breaking up into fragmented masses. The presence of 0.1 per cent NaCl prevented plasmoptysis, but the bodies became round, and the flagella rigid and swollen. In a 0.2 per cent NaCl solution the organisms were slightly swollen, but motile. A concentration of 0.3 to 0.9 per cent NaCl preserved Leishmania in excellent condition. Half-saturated NaCl made them appear rigid, shrunken, and refractile.

Acid and Alkali.—HCl, added to a 0.9 per cent NaCl in a concentration of N/1 to N/10 promptly inhibited the motility of Leishmanias, the cells becoming highly refractile, but retaining the flagellum.

NaOH, added to 0.9 per cent NaCl in a concentration of from N/1 to N/10 caused cessation of motility, the cell bodies became indistinct, and in most instances the flagella dissolved within a few minutes. In 0.9 per cent NaCl with a pH of 8.2, the Leishmanias were all active.

All the strains grew well in medium with a pH of 5.08 to 7.21, but *L. tropica* grew well up to pH 8.8 and *L. infantum* up to pH 8.19. Rich growth of *L. brasiliensis* occurred at pH 7.7 and some growth at pH 8.19 but none beyond. *L. donovani* did not grow beyond pH 7.21.

· *Chemical and Biological Agents.*—Saponin in dilutions of 1:10 to 1:10,000 killed the Leishmanias without dissolving them. Sodium taurocholate dissolved them in 1:100, but in 1:1,000 dilution a few organisms still survived. Sodium oleate destroyed them in 1:100 but not completely in 1:1,000. Ricin, dissolved in saturated NaCl 1:10, then diluted to 1:100 with distilled water to render the mixture isotonic, showed a definite immobilizing and clumping effect upon Leishmanias, while abrin of similar concentration had no perceptible effect. In higher dilutions ricin was ineffective.

Snake venoms, themselves of animal origin, were also tested for their effects upon Leishmania. The venoms of *Crotalus adamanteus* (Florida rattlesnake), *Ancistrodon piscivorus* (water moccasin of the United States), *Lachesis flavoviridis* (*habu* of Formosa), *Lachesis lanceolatus* (South America), *Vipera russelli* (daboia of India), and *Vipera berus* (*mamushi* of Japan) exerted no effect upon the Leishmanias when used in a 1:200 dilution. On the other hand, the venom

of the cobra (*Naja tripudians* of India) caused immobilization in 1:200 and 1:2,000, while the addition of lecithin rendered the cobra venom active against Leishmania in a 1:20,000 dilution; in venom of this concentration the organisms remained active until the lecithin was added. The activation by lecithin was also noticeable with the venoms of *L. lanceolatus*, *L. flavoviridis*, *Ancistrodon piscivorus*, and daboia, but scarcely at all with the venoms of the rattlesnake and the little Japanese viper. The leishmaniolysis by certain snake venoms in the presence of lecithin is analogous to the hemolysis occasioned by the same combination, and the comparative innocuousness of various snake venoms upon a protozoan organism is a point of interest.

Effect of Chemotherapeutic Preparations.

The effectiveness of tartar emetic in the treatment of leishmaniasis appears to be well established.[6] The exact manner by which these antimony compounds act upon the parasites in the human body is not known; hence it was of theoretical interest to inquire into the mechanism of the action of these drugs by way of test-tube experiments, of which 3 types were made: (1) the direct effect of drugs *in vitro* upon Leishmania; (2) the effect of the same drugs after being mixed with fresh animal-tissues (rabbit-liver or kidney) *in vitro*; and (3) the effect of the drugs after having been introduced into the animal body, as determined by examining *in vitro* the effect of the serums derived from the drugged animals.

Of the antimony compounds, tartar emetic was dissolved in 0.9 per cent NaCl in concentration of 1:100. From these stock solutions further dilutions, corresponding to 1:1,000, 1:10,000, and 1:100,000 were prepared, and active Leishmania cultures were added to each. Tartar emetic in 1:100 solution killed the organisms promptly, but in a 1:1,000 dilution some were still alive after 1 hour, and a 1:10,000 dilution had no effect. Emetine hydrochloride killed the organisms in 45 minutes in 1:1,000 dilution, but not in 1:10,000 dilution. The addition of fresh rabbit-kidney or liver emulsion to these drugs neither increased nor decreased their inherent toxicity for Leishmaina:—that is, there was no immediate transformation of the

[6] WENYON, C. M.; *Trop. Dis. Bull.*, 1922, xix, 5, 185.

drugs into a more toxic form by the action of fresh somatic cells *in vitro*.

Eight bismuth compounds, the tartrate, albuminate, citrate, oxalate, lactate, benzoate, subgallate, and acetate, were similarly studied, but their insolubility in water render the determination of their inherent germicidal action *in vitro* highly uncertain. They were inactive when used as suspended particles. Attempts to transform these substances into toxic form by grinding them with a fragment of fresh rabbit-liver were not uniformly successful, the tartrate being the only one which acquired a slight toxic effect after this treatment. The emulsion containing the tartrate in ratio of 1:100 had a definite injurious action upon the Leishmanias, but a ten-fold dilution of it exhibited only a slight inhibition of motility.

Ten arsenic compounds, including salvarsan, neosalvarsan, tryparsamide,[1] and A 96,[7] were similarly tested. In direct action *in vitro* salvarsan and neosalvarsan killed the organisms in a 1:1,000 dilution and immobilized many in 1:10,000 within 2 hours, while tryparsamide and A 96 affected them only slightly in a 1:200 dilution. Salvarsan was used in a slightly alkaline solution (pH 7.4); the others were neutral and required no adjustment of reaction. Several arsenic compounds which were available for this series of experiments were omitted because they had to be dissolved by adding NaOH, and it had already been found that Leishmania was sensitive to any slight excess of alkalinity or acidity. Salvarsan and neosalvarsan treated with fresh rabbit-liver *in vitro* did not acquire a greater toxicity for Leishmania. A dilution of the salvarsanized liver suspension corresponding to 1:1,000 of the drug killed the Leishmanias promptly, but not the 1:10,000. It may be recalled here that the original germicidal titre of salvarsan for *Treponema pallidum* (culture) was between 1:1,000 and 1:10,000, but that it rose to 1:100,000 after treatment with fresh rabbit-liver.

As already stated, all the bismuth compounds and several arsenic preparations were insoluble in water, but there was a possibility that some of these drugs might become soluble and reveal their germicidal

[7] I am indebted for these drugs to Dr. Wade H. Brown, of the Rockefeller Institute for Medical Research.

action when injected into the veins of certain suitable animals—for example, rabbits. Five of the arsenic benzene derivatives might be expected to undergo such a change *in vivo*, since they dissolved *in vitro* upon addition of NaOH in adequate quantities. Each of 24 rabbits received a suspension or solution of one of the 23 substances tested. In the case of tartar emetic and emetine hydrochloride, a 2 per cent aqueous solution was used.

A rabbit weighing 1,900 grams received 8 cc. of 2 per cent tartar emetic solution into the marginal ear vein, and died within 4 minutes after severe reactions. The blood was withdrawn from the heart 9 minutes from the time of injection. A rabbit of 1,550 grams receiving 2 cc. of 2 per cent tartar emetic survived, though reacting severely for several minutes; the blood was collected 1 hour after injection; 2 cc. of a 2 per cent emetine solution were given intravenously to a rabbit weighting 1,800 grams, the animal died within a few minutes, and the blood was taken from the heart.

The serum from the first rabbit had no effect upon Leishmania, but the serum from the second caused a decided slackening of motility in 1:10 dilution; the serum from the third rabbit (emetine) immobilized the organisms in a 1:10 dilution. The 1:10 dilution was chosen for the tests of the drugged serums, because normal rabbit serum killed Leishmanias when undiluted, but had no effect upon them in a 1:10 dilution.

All the bismuth salts were suspended in saline in concentration of 0.1 gram per 10 cc. of saline, but the quantities injected could not be exactly estimated because of the rapid sedimentation of the insoluble particles while the syringe was being charged. Each animal received approximately 0.05 grams intravenously, and none died within 1 hour. At the end of this period they were sacrificed, to obtain serum. A slight diminution of motility was noticed when cultures of Leishmania were mixed with the serums derived from rabbits injected with the tartrate and subgallate of bismuth, but no injurious effect was perceptible with the serums from animals receiving the other bismuth compounds. Ten organic compounds of arsenic tested similarly yielded chiefly negative results. Salvarsan and neosalvarsan contained in the serum of rabbits intravenously injected with 0.1 and 0.15 grams respectively, proved to be highly germicidal for *Treponema*

pallidum and *Spironema dultoni*, notwithstanding their inertness toward Leishmania, showing their specific affinity for spirochetes.

Sodium oleate and urotropin, 0.1 gram given intravenously to rabbits, did not yield a serum germicidal for Leishmania. The oleate killed the animal in 3 minutes.

The foregoing experiments left the mechanism of the curative effect of antimony treatment in human leishmaniases unsolved. It is striking that tartar emetic and the bismuth compounds are so slightly injurious for Leishmania, either directly or indirectly through the action of fresh tissues or the animal body. A greater germicidal effect on Leishmania was exhibited by salvarsan and neosalvarsan, and was retained after fresh tissues or the animal body had acted upon them, while the spirocheticidal potency of these two drugs was greatly enhanced by passage through the animal body. It seems, therefore, that the curative effects of these compounds in the treatment of human leishmaniases are brought about by a slow transformation of comparatively insoluble substances into a soluble parasiticide in the human body.

Dyes and Photodynamic Sterilization.

During the study of various disinfectants, including some of the germicidal dyes, a very striking phenomenon of photodynamic sterilization was encountered. A dye known as neutral acriflavine, or, more simply, "neutroflavine,"[8] a derivative of acid acriflavine (3, 6 diamido-10-methyl acridinium) was found to be markedly germicidal for Leishmania when employed in sufficient concentration, 1:50,000. But beyond a certain point, it was no longer germicidal, yet these higher dilutions exerted a rapid devitalizing effect when the dark-field microscope was used to examine the preparations. A suspension of active Leishmania culture, for example, in a dilution of 1:100,000 of neutroflavine remained unaffected in ordinary diffuse daylight in the laboratory, for many hours; but the moment a slide prepared from the mixture was brought to focus under the dark-field microscope (a Leitz arc-lamp being used), the actively motile flagellates lost their motility instantaneously. The effect was so striking as

[8] Made by the National Aniline Company, 40 Rector St., New York City, and sold at a price of \$6.00 for 5 grams.

to suggest the so-called photodynamic action of certain fluorescent dyes, but when actually tested none of the fluorescent photosensitizers, such as erythrosin, eosin, and fluorescein, showed any germicidal property, either with or without the aid of the arc-lamp. Cyanine, a blue sensitizer, showed marked germicidal and photodynamic action somewhat comparable to neutroflavine, but the dye was difficult to work

TABLE 1.

Dyes and Photodynamic Sterilization.

	Native Germicidal Concentration Minimum	Range of Photodynamic Concentrations	
		Optimum	Minimum
Neutroflavine	1:50,000	1:100,000	1:10,000,000
Neutral red	1:50,000	1:100,000	1:1,000,000
Brilliant green	1:100,000	1:1,000,000	1:10,000,000
Janus green	1:100,000	1:1,000,000	1:10,000,000
Gentian violet	1:1,000,000	1:5,000,000	1:10,000,000
Tryparosan	1:10,000	1:100,000	1:1,000,000
Basic fuchsin	1:10,000	1:100,000	1:1,000,000
Acid fuchsin	None at 1:1,000	None at any dilution	
Pyoktanin blue	1:100,000	1:1,000,000	1:10,000,000
Brilliant blue	1:1,000	1:5,000	1:10,000
Pyrrol blue	1:10,000	1:100,000	1:1,000,000
Cyanine	1:50,000	1:100,000	1:1,000,000
Optochin	1:5,000	None at any dilution	
Trypan blue	None at 1:100	None at any dilution	
Trypan red	None at 1:100	None at any dilution	
Scarlet red	None at 1:100	None at any dilution	
Erythrosin	None at 1:100	None at any dilution	
Eosin	None at 1:100	None at any dilution	
Fluorescein	None at 1:100	None at any dilution	
Phenol	1:100	None at any dilution	
$HgCl_2$	1:50,000	None at any dilution	
H_2O_2 (30%)	1:1,000	None at any dilution	
Formalin (40%)	1:1,000	None at any dilution	
Lugol's solution	1:100	None at any dilution	

with in this sort of experiment because of its practical insolubility in water. A brief experimental survey of various dyes furnished the following data (Table 1).

Of 18 dyes examined, 11 proved to be capable of exerting a photodynamic destructive action upon Leishmanias. In the majority of

cases the optimum photodynamic doses were close to the minimum native killing doses.[9] The minimum photodynamic doses, however, were much smaller with certain dyes than with others. In the case of neutroflavine it was 1:2,000 of the native disinfecting dose, while with brilliant green, Janus green, basic fuchsin, tryparosan, pyoktanin blue and pyrrol blue, it was 1:100, and with gentian violet and brilliant blue it was only 1:10. The great native disinfecting power of gentian violet against Leishmania (1:1,000,000) was extraordinary as compared with our everyday disinfectants such as phenol, mercury bichloride, hydrogen peroxide, formalin, and iodine, yet Leishmania is Gram-negative.

Whether or not the same atomic groups of a photodynamically active dye are responsible for the native disinfecting action in higher concentrations and the photodynamic effect in greater dilutions, I cannot say, but the following observations seem to have some bearing on the question:—Neutroflavine presents a deep brownish-black color at a 1:100 dilution, a deep brownish-yellow at 1:1,000, a bright orange at 1:10,000, a daffodil-yellow at 1:100,000, a pale lemon at 1:1,000,000, a trace of yellow at 1:10,000,000, and no perceptible color at 1:100,000,000. Solutions of from 1:100 to 1:50,000 concentrations (still bright yellow) kill the flagellates in a laboratory room (diffuse daylight) like any ordinary disinfectant, but those from 1:100,000 to 1:10,000,000 concentration (trace of yellow) kill them only in the presence of a powerful light. The 1:100,000,000 dilution is not strong enough to have any action, even in the presence of intense rays. The potentially photodynamic solutions of the dye behave very differently, according to the variety and intensity of the rays used. For example, rays passed through a blue filter kill the Leishmania suspended in a 1:1,000,000 dilution of neutroflavine within 1 to 2 seconds, green rays within 10, and greenish-yellow within about 30 seconds, while rays passed through an orange, yellow, or red filter are without effect.

It is obvious that only rays of shorter wave-length are concerned in this phenomenon. Direct sun's rays applied to a slide preparation of

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[9] In the case of neutroflavine, there is a zone of concentration in which there is no native germicidal effect, yet the dye is too concentrated (perhaps too yellow) to become photodynamically active.

Leishmania suspended in a 1:1,000,000 solution of neutroflavine kill the organisms in about 30 seconds, but by condensation of the rays upon the slide (which is protected from heat by means of cooling devices), killing can be accomplished in a few seconds. Leishmanias in control-slides, minus the dye, stand all these manipulations perfectly well and remain active for a long time. That the acquisition of the anti-Leishmania property by the very high dilutions of neutroflavine was not due to the action of heat, was indirectly shown by the filtered-ray experiments and by the application of various temperatures (26° to 37°C.) in the absence of light. No evidence has been obtained that the formation of a toxic substance from a dilute solution of neutroflavine by the action of actinic rays takes place in the absence of living cells (Leishmania, Treponema, Spironema, and Leptospira). The photodynamic action takes place only in a mixture of susceptible organisms and dilute dyes in the presence of sufficiently intense rays.

Neutroflavine is relatively non-toxic for man, has a strong inherent antiseptic property, and, finally, the unusually powerful photodynamic sterilizing quality in a dilution as high as 1:10,000,000 in the presence of actinic rays.

That neutroflavine remains active in the blood for the first 2 hours after its introduction into the general circulation, has been proved in an experiment upon a rabbit. The animal received 10 mg. neutroflavine per kilogram body-weight intravenously, and specimens of the blood were tested after 10, 45, and 75 minutes, after 2 hours, and finally after 20 hours. All specimens except the last, which was without any action, contained enough neutroflavine to tint the citrate plasma yellowish, and revealed a prompt photodynamic action on Leishmania.

Neutroflavine might first be tested for its therapeutic action as an external disinfectant over ulcerated lesions in a 1:1,000 solution, and simultaneously as a local tissue-sterilizer, by injection of a 1:10,000 solution into the lesions, but greater benefit might be derived from its use as an internal (in cases of visceral leishmaniasis) and also as a photodynamic disinfectant. To attain the concentration of 1:50,000 (the minimum native disinfecting dose) of the dye in the general circulation of a person having approximately 6,000 cc. of blood (esti-

mating the blood as 1/13 of 80 kilograms body weight), 0.12 gram of the substance would have to be given intravenously, an amount only ¼ of that which the rabbit tolerated without inconvenience. In Germany neutroflavine has been used in a dose of 0.2 gram at 12-hour intervals (intravenously) in cases of influenza, pneumonia, and septicemia, without any ill effects. Neutroflavine is eliminated through the urine at the rate of 0.2 gram in 36 to 48 hours, according to a report from Germany, where the dye has been used in large quantities for the treatment of acute infections.

X-rays, unlike actinic rays, are unable to convert neutroflavine into a substance toxic for Leishmania, and are themselves inactive toward the organism.

SUMMARY AND DISCUSSION.

Leishmania donovani, *L. infantum*, *L. tropica*, and *L. brasiliensis* grow well on a semi-fluid medium described and form a heavy, grayish surface-growth several millimeters in depth. The medium is easily preserved and facilitates maintenance of subcultures, isolation of new strains, and employment of large quantities of the organisms.

All of these strains of Leishmania require oxygen for growth. None was able to grow in an atmosphere of hydrogen, nitrogen, or ·carbon dioxide.

All the strains of Leishmania studied grew well when the hydrogen ion concentration of the medium was within the range of pH 5.08 to pH 7.21, but *L. tropica* and *L. infantum* grew well up to pH 8.8 and pH 8.19, respectively, while *L. brasiliensis* and *L. donovani* did not grow beyond pH 8.19 and pH 7.21, respectively.

All the strains studied were killed by an alkalinity greater than N/10 NaOH or an acidity greater than N/10 HCl, when the acid or alkali was added to 0.9 per cent NaCl. Distilled water causes immediate disintegration of the flagellates, while a tonicity greater than 0.3 per cent and up to 0.9 per cent NaCl is well-borne. The organisms are immobilized by half-saturated saline solution.

Certain phytotoxins and plant toxalbumins kill Leishmania cultures. For example, saponin in a 1:10,000 dilution killed them without dissolution of either the bodies or the flagella. Ricin in a 1:100 dilution caused immobilization and agglutination, but abrin was inactive.

Leishmania was also killed by a number of animal poisons under certain conditions. The venom of the Indian cobra affected them in a 1:2,000 dilution in 0.9 per cent NaCl and killed in a 1:20,000 dilution when a small amount of lecithin (otherwise harmless) was added. Solutions in saline (1:200) of the venoms of *Lachesis lanceolatus*, *L. flavoviridis*, *Ancistrodon piscivorus* (water moccasin), *Crotalus adamanteus* (diamond-back rattlesnake of Florida), *Vipera russelli* (Indian daboia), and a Japanese viper (*mamushi*) had no effect upon Leishmania *in vitro*. In the presence of lecithin, however, the venoms of Lachesis and Ancistrodon exerted a slight lytic effect, but those of the rattlesnake and *mamushi* remained inactive.

By means of monovalent immune serums produced in rabbits it was possible to differentiate, through agglutination tests, and cultivation on media containing immune serums, *Leishmania donovani* from *L. tropica* or *L. brasiliensis*, each of these strains representing a serologically independent and distinct unit. *L. infantum* was found to be serologically identical with, or closely allied to, *L. donovani*. These findings conform with the clinical observations, which indicate that the visceral leishmaniases (*L. donovani* and *L. infantum*) are distinct from the benign oriental sore (*L. tropica*), which is merely a skin infection, and probably also from the American type of leishmaniasis (*L. brasiliensis*), which involves both skin and mucous membranes and is often malignant. This distinction may prove useful in the utilization of immunity phenomena in connection with prophylactic and therapeutic measures.

Attempts were made to determine the mechanism by which tartar emetic acts to effect a cure in leishmaniasis. The antimony compound was found to be only slightly germicidal for Leishmanias *in vitro*, a 1:100 solution being required to kill them. Brief contact with fresh animal-tissues, or intravenous introduction into rabbits, did not transform this substance into a more potent germicide for these organisms. Hence the exact mode of action of the drug upon the parasites in human leishmaniasis has not been explained.

Salvarsan, sometimes reported to have cured leishmaniasis, and also neosalvarsan, have been similarly studied, and both showed a native disinfecting power nearly 10 times as great as that of tartar emetic. A 1:1,000 solution of salvarsan killed the Leishmanias in

saline solution, and both arsenical compounds retained their germicidal powers after having been emulsified with a fragment of fresh rabbit-liver or subjected to the action of the animal body by means of in-travenous inoculation into rabbits. These procedures, however, greatly enhanced the germicidal power of these two drugs for *Treponema pallidum* and *Spironema duttoni*.

Several other organic compounds of arsenic and bismuth have been studied in the same way, but the results were even less informing. Bismuth tartrate seemed to acquire slight leishmanicidal power after treatment with fresh tissues or injection into the animal body.

The photodynamic properties of certain fluorescent dyes have long been known, and fluorescence and photodynamic action were thought to be closely associated. In the present study, however, a peculiar phenomenon was observed, in which flagellates and spirochetes were rapidly killed by extraordinarily dilute, and otherwise inactive, so-lutions of certain germicidal dyes in the presence of actinic rays. Neither the solutions nor the rays alone harmed the organisms, and the dyes did not seem to have been converted into a germicide of greater potency, since solutions exposed to the rays without the simul-taneous presence of the microorganisms did not become germicidal.

The occurrence of the phenomenon required the simultaneous presence of the dye in high dilutions, the microorganisms, and actinic rays, and is therefore somewhat different from the so-called photo-dynamic action of certain fluorescent dyes in which the formation of peroxide in the presence of ordinary light is said to play a part. The phenomenon does not occur with any of the well-known photo-dynamic fluorescent dyes—eosin, erythrosin, and fluorescein—none of which was either inherently or photodynamically germicidal for the flagellates. Eleven of 18 dyes, chiefly non-fluorescent, were found to possess native, as well as photodynamic, germicidal properties. The most striking example of the group is neutral acriflavine or neutro-flavine, which killed Leishmania, Treponema, Spironema, and Lep-tospira in a dilution of 1:50,000 without the aid of a special light, and in a dilution of 1:10,000,000 with the aid of actinic rays. An arc-lamp or the sun's rays furnish all the actinic energy required for this action. Rays filtered through a red, orange, or yellow screen exert no photodynamic action upon the dye solution, but those passed through a blue filter act most energetically.

The fact that neutroflavine is well tolerated by man, remains in the circulation active for many hours, possesses a strong inherent antiseptic property, and, above all, the unusually powerful photodynamic sterilizing quality in a dilution as high as 1:10,000,000, makes it highly promising as an agent for the treatment of certain protozoan diseases associated with chronic ulcers. Leishmaniasis and various forms of spirochetosis offer a wide field for testing out the sterilizing effect of this and allied dyes in tropical regions.

ADDENDUM.

Plant Flagellates and Leishmania.

The various skin ulcers of Brazil, particularly those of the Brazilian type of leishmaniasis, are said to be very common among persons who are engaged in clearing uncultivated woodlands, and the possibility suggests itself of demonstrating the pathogenic Leishmania in plants. A great deal of investigation[10] regarding the natural reservoirs of Leishmania in plants and invertebrates has already been carried out by Lafont, França, Laveran and Franchini, Rhodain and Bequaert, Noc and Stévenel, Léger, Migone, Fantham, Hoare, Glaser, and Becker, but there have been only occasional successes in transmitting the flagellates of plants or insects to a mammalian host. Strong,[11] in a recent important contribution to this subject, reports that he has found flagellates morphologically identical in the latex of Euphorbia, in a hemipteran insect feeding upon the plants, and in the intestine of a lizard probably feeding on the insects, and that he has succeeded in producing a skin ulcer in a monkey by injection of flagellate-containing

[10] LAFONT, A.; *Bull. Soc. Path. Exot.*, 1911, iv, 464. FRANÇA, C., *Arch. f. Protistenk.*, 1914, xxxiv, 108; *Bull. Soc. Path. Exot.*, 1911, iv, 532, 669; *Ann. de l'Inst. Pasteur*, 1920, xxxiv, 432. LAVERAN, A., and FRANCHINI, G.; *Bull. Soc. Path. Exot.*, 1921, xiv, 148. RHODAIN, J., and BEQUAERT, J.; *Bull. Soc. Path. Exot.*, 1911, iv, 198. NOC, F., and STÉVENEL, L.; *Bull. Soc. Path. Exot.*, 1911, iv, 461. LÉGER, A.; *Bull. Soc. Path. Exot.*, 1911, iv, 626. MIGONE, L. E.; *Bull. Soc. Path. Exot.*, 1916, ix, 356. FANTHAM, H. B.; *Ann. Trop. Med. and Parasitol.*, 1915, ix, 341; *S. African Journ. Sci.*, 1922, xix, 332. HOARE, C. A.; *Parasitology*, 1921, xiii, 67. GLASER, M.; *Journ. Parasitol.*, 1922, viii, 99. BECKER, E. R.; *Am. Journ. Hyg.*, 1923, iii, 462.

[11] STRONG, R. P.; *Am. Journ. Trop. Med.*, 1924, iv, 345.

intestinal contents of the lizard. Non-flagellated forms of the pro-
tozoön were found in the granulomatous tissues at the site of the ulcer,
forms morphologically similar to the aflagellate form of Leishmania.

During my brief sojourn in Kingston, while attending the present
Conference, I had the good fortune to meet several investigators who,
like myself, were interested in plant flagellates, and through the kind
advice of Dr. Juan Iturbe, of Caracas, Venezuela, and Dr. R. W.
Hegner, of Johns Hopkins University, I was able to begin a brief
study, the results of which follow. My interest in this subject was
limited to obtaining the flagellates in culture, if possible, and com-
paring their morphological, biological, and immunological properties.

Two varieties of Euphorbia found in Kingston (*E. pilulifera* and *E.
maculata* (?)) and about a dozen latex plants in the Hope Botanical
Garden[12] were examined, with negative results.[13] The search was
renewed in Tela, Honduras, where Strong had recently demonstrated
flagellates in *Euphorbia pilulifera* and Hegner in *Asclepias curassavica*,
and with the help of Dr. Herbert C. Clark, of Tela Hospital I was able
to obtain material for study. Among about 50 milkweeds (*Asclepias
curassavica*) (Figs. 9, 10) flagellates were found in the latex of 2; and
of nearly 100 *E. pilulifera*, and *E. (Chamaesyce) brasiliensis* (Fig. 11)[14]
only 1 of the former and 2 of the latter proved to be infected. In
accordance with Hegner's previous observation, the older Asclepias
with pods were the ones infected, and in the case of Euphorbia, the
flagellates were found in old, sickly-looking specimens, as recorded by
Lafont, França, and Strong. A number of specimens of the creeping
variety of Euphorbia (*E. maculata* (?)) were examined with negative
results.

In all of the 5 positive plants, the infection was confined to certain
stems or branches; for example, only 1 of 2 collateral branches would
contain the flagellates. The number of organisms present in the

[12] For the use of the dark-field microscope I am indebted to Drs. MacLean and
Moody, of Kingston.

[13] About 50 specimens of Euphorbia, collected in the suburbs of Havana and
on the grounds of Las Animas Hospital on the occasion of my visit to Dr. Lebredo,
the Director, and brought back to New York, were also negative.

[14] For identification of these specimens of Euphorbia (Chamaesyce) I am
indebted to Dr. H. A. Gleason, of the New York Botanical Garden, Bronx Park.

infected latex is astonishingly large, each field (objective 2 mm. and ocular K. 8) revealing a dozen or more actively motile organisms.

As also observed recently by Holmes,[15] the American milkweed, *Asclepias syriaca* (probably *syriaca*) also occasionally harbors a flagellate of the herpetomonad type. Several infected plants have been encountered among several hundred examined on Long Island, N. Y.

On the flowers and pods of *Asclepias curassavica* were found numerous nymphs and winged adults of a hemipteran insect, later identified by Dr. H. G. Barber, of the American Museum of Natural History, as *Oncopeltus cingulifer* (Fig. 12). Six of the nymphs brought back from Honduras were dissected at the Rockefeller Institute by Miss Tilden, who found numerous herpetomonads in the intestines of 3 of them. Four adults, 1 male and 3 females, were placed in a cage on a group of the *Asclepias curassavica* in a greenhouse (80°F.); 2 weeks later a cluster of 80 salmon-pink eggs (about 0.8 mm. in diameter) were found in a crevice of the cage. These hatched 4 days later, and in 3 weeks the nymphs passed through several moultings and attained a size of about 5 mm. Five of these were examined at this period, but in only 1 was the herpetomonad found. Later on, however, practically all were found to be infected with the herpetomonad. The plants upon which the adults and young nymphs have been feeding for more than 3 months have gradually lost most of their young leaves but repeated examinations of leaves from these plants has so far failed to show that the latex is infected with flagellates.

Morphology of the Flagellates.

In a freshly prepared slide, as seen by dark-field illumination, the plant forms are rather sluggishly motile, and many of them are provided with a flagellum. The body is an elongated, thin, lanceolate form with smooth contour, narrowing at both extremities to a point; one end is somewhat less pointed than the other, and the flagellum is attached at this end. From the posterior end a thin tremulous filament is often seen, which appears as if it were drawn out of some viscid substance such as mucin. The flagellum moves in a wriggling or jerking fashion,

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[15] HOLMES, F. O.; *Phytopathology*, 1924, xiv, 146.

drawing the otherwise immotile body forward. The comparative shortness of the flagellum and the flattened body are in striking contrast to the usual culture forms of various strains of Leishmania, as seen by dark-field illumination.

At the end of about an hour the number of motile forms in the preparation becomes fewer, and there appear a great many immotile organisms devoid of flagella, which tend to twist spirally once, twice, or even half a turn more. Occasionally these forms are seen in fresh preparations.

Stained with Giemsa's or Wright's solution, the herpetomonad from *Asclepias curassavica* (Fig. 6) is shown to have an oblong or round nucleus and a short, rod-shaped parabasal body transverse to the axis of the body, and close to the attachment of the flagellum. The organism is somewhat shorter and broader than the one found in *Euphorbia brasiliensis* (Fig. 5) the length varying from 14 to 20 μ, and the width from 2 to 4 μ, as compared with a length of 17 to 30 μ and a width of 1.4 to 2 μ for the Euphorbia organism. The distance between parabasal body and nucleus is also less, averaging 1.9 μ as compared with 3.8 μ. In the Euphorbia flagellate the parabasal body is round. The flagellate found in *Asclepias syriaca* (Fig. 7) is very similar to that of *A. curassavica*.

The herpetomonad found in the intestine of *Oncopeltus cingulifer* (Fig. 8) as seen by dark-field examination, is more actively motile than those of the plant latex. The long flagellum—its length usually exceeds that of the body—moves in a serpentine fashion. The body is somewhat longer and thicker, and shows less tendency to spiral twisting, and the cytoplasm contains a number of highly refractile granules, rarely seen in the plant forms. In preparations stained with Giemsa's solution the length of the body varies from 18 to 28 μ, average 24 μ, the width at the widest portion being 1.8 to 3.5 μ, and the distance from parabasal body to nucleus 3 μ. The parabasal body is round and measures about 1 μ in diameter.

There is little or no difficulty, either in fresh or stained preparations, in distinguishing the insect from the plant forms. Some specimens of the herpetomonad forms of Leishmania, grown on leptospira medium, are very similar to the insect forms, but in general the Leishmania flagellates (Figs. 1–4) are shorter and have a thicker, and often broader,

body. In the culture forms of Leishmania, moreover, the length of the flagellum rarely exceeds that of the body, and in pyriform specimens the nucleus is situated near the posterior end of the body. For comparison there are recorded here the measurements of the plant and insect forms. Attempts were made to record similar measurements for Leishmania, but the dimensions of the multiplying culture forms vary greatly, not only in cultures of different ages, but in the same culture, while those of the insect and plant forms are fairly constant; hence the measurements would not be comparable.

TABLE 2.

Herpetomonads from Asclepias curassavica.

	Length of Body	Anterior End to Parabasal Body	Parabasal Body to Nucleus	Nucleus	Posterior End to Nucleus	Width at Widest Portion	Length of Flagellum
Methyl alcohol fixation	20μ	2μ	1.8μ	2.8μ	13μ	3μ	5μ
	19	1.8	1.6	3	13	4	
	17	2	1.5	3.2	10	2.5	2
	17	1.8	2.2	2.5	10	2.8	
	15	1.2	1.5	2.5	9	3	
	16	1.5	2	2.2	9	3	
	20	1.6	2	2.5	14	2.5	
	15	1.8	1.4	2.2	9	3	
	16	1.5	2	2	10	2.5	
	20	1.8	2	3	13	2.8	
	15	1.2	1.8	2.2	9	2.6	
	20	1.5	1.7	2.5	14	2.8	
	15.2	1.5	1.5	2.1	10	2.3	
	17	1.5	1.5	1.5	12	2.3	
	20	2	1.8	2.6	12	2.4	3
	20	2	1.8	2.5	13.5	4	12
	18	2	1.5	3	11	3	
S. A. fixation	17	1.8	1.5	3	10	3	
	16	1.5	1.3	2.5	10	3	7
	16	1.8	2	2.6	9.4	2.5	9
	14	1.5	1.5	2	9	2	3
	16	1.5	4	2	8.5	2	
	16	1.5	4	2.5	8	2.2	
	17	1.5	2.5	2	11	2	
	17	1.5	2.5	2.5	10.5	2.1	
Range.......	14–20μ	1.2–2μ	1.3–4μ	2–3μ	8–14μ	2–4μ	3–12μ
Average.....	17.5μ	1.7μ	1.9μ	2.5μ	10.7μ	2.7μ	

Whether or not the herpetomonads found in the latex plants and in the hemipteran insects associated with them are identical, and only modified by the difference in hosts, or represent more than one species, as would appear from purely morphological features, is yet to be determined.

Cultivation.

The facility with which a strain of human Leishmania can be cultivated in the semi-fluid leptospira medium, led to its being the first tried for the cultivation of the plant flagellates. Both human and

TABLE 3.

Herpetomonads from Euphorbia brasiliensis.

Length of Body	Anterior End to Parabasal Body	Parabasal Body to Nucleus	Nucleus	Posterior End to Nucleus	Width at Widest Portion	Length of Flagellum
24μ	2μ	4μ	3μ	14μ	1.5μ	10μ
21	1.8	2.2	2	14	1.8	12
17	1.6	2.5	2.2	11.3	1.4	12
24	1.8	2.1	2.5	17.6	1.8	12
19	1.8	2	2	13.2	1.8	12
28	1.5	4	2	20	1.8	15
22	1.5	5	2.2	13.3	1.8	12
22	1.5	5	2.2	13.3	1.8	10
30	2	4	3	21	2	15
25.2	2	5	2.2	16	2	14
30	1.8	6	3	19.2	2	14
Range...17–30μ	1.5–2μ	2–6μ	2–3μ	11.3–21μ	1.4–2μ	10–15μ
Average.23.8μ	1.75μ	3.8μ	2.4μ	15.7μ	1.8μ	12.5μ

rabbit serum and hemoglobin were alternately used in its preparation. In addition to the usual medium with alkaline reaction (pH 7.5), media adjusted to various degrees of alkalinity and acidity were employed, including one made acid to a degree approximating the reaction of the latex of Asclepias and Euphorbia. The addition of N/10 HCl in quantities greater than 0.3 cc. to 8 cc. of the regular leptospira medium causes opalescence and finally heavy precipitation of the serum constituents. Fresh and also autoclaved coconut milk, the acidity of which is approximately the same as the plant latex, were used both with and without the addition of serum, hemoglobin, or

agar. The regular N. N. N. blood agar slant was also tried. The inoculum consisted of the fresh latex, which oozes out when a leaf is

TABLE 4.

Herpetomonads from Asclepias syriaca.

Length of Body	Anterior End to Parabasal Body	Parabasal Body to Nucleus	Nucleus	Posterior End to Nucleus	Width at Widest Portion	Length of Flagellum
18.5μ	2μ	4μ	2.5μ	10μ	2.2μ	8μ
14	1.8	4	2.2	6	2.2	8
16	2	3	2	9	2.2	10
18	2	4	2.2	10	2.5	10
18	2	3.5	2.3	11	3	10
17	2	3	2.2	10	3.5	10
20	1.8	2.5	3	12	2.5	10
20	2	4	2.5	11	2.5	12
16	1.8	4	2.1	8	2.8	8
18	1.8	2.2	2.2	12	2.5	10
22	2	2.5	3	14.5	3	12
13	1.8	2.5	2.8	5	2.5	6
15.5	2	3.5	3	7	2.2	12
13	2	2	3	6	3.2	6
14	1.8	4	3	6	3.5	9
22	2	5	3	12	2.2	12
11	1.5	2.2	2.2	5	2	6
20	2	1.2	2.2	12.5	2.5	6
16	1.8	2	2.5	10	2	8
16	1.8	2.2	2.2	10	2.5	12
Range...13–22μ	1.5–2μ	1.2–5μ	2–3μ	5–14.5μ	2–3.5μ	6–12μ
Average.16μ	1.9μ	2.8μ	2.5μ	9μ	2.3μ	8.8μ

TABLE 5.

Herpetomonads from Oncopeltus cingulifer.

Length of Body	Anterior End to Parabasal Body	Parabasal Body to Nucleus	Nucleus	Posterior End to Nucleus	Width at Widest Portion	Length of Flagellum
25μ	4μ	3μ	3μ	15μ	3.5μ	28μ
24	4	3	3	14	3	25
18	2	3	2.5	7	2	18

broken off. Bacterial contamination almost invariably followed the use of undiluted latex, but when the latex was diluted 1:10 or 1:20

with sterile saline or Ringer-Tyrode not containing bicarbonate, the frequency of bacterial contamination was greatly reduced. Cultivation was carried out at laboratory temperature in Tela, the cultures were kept in the steamer cabin during the trip from Honduras to New York, and the temperature has not exceeded 35°C. at any time.

No culture of the plant flagellates has thus far been obtained. The longest survival was 12 days on the leptospira medium, but with no indication of multiplication. On the other hand, cultures of *L. tropica* and *L. brasiliensis* grew luxuriantly, under the same conditions, on the leptospira medium and also on the N. N. N. medium.

Attempts to cultivate the herpetomonads of *Oncopeltus cingulifer* have likewise been fruitless. It is evident, therefore, that the cultural requirements of the plant and insect flagellates studied are quite different from those of Leishmania.

Effects of Anti-Leishmania Immune Serums upon the Plant and Insect Flagellates.

Although cultures could not be obtained, the latices of the plants and the intestinal contents of *Oncopeltus cingulifer* constituted natural cultures, owing to the extremely large numbers of the motile flagellates present. It appeared feasible, therefore, to utilize these materials to determine the action of the immune serums of high titre which had been produced by immunization of rabbits against the various strains of Leishmania. Four types of immune serums were used, anti-*brasiliensis*, anti-*tropica*, anti-*infantum*, and anti-*donovani*.

The results of this experiment were decisive, inasmuch as neither the plant nor the insect flagellates were influenced by the addition of any of the 4 anti-Leishmania immune serums, their motility and form remaining unchanged even in dilutions of 1:2 and 1:20. There was a phenomenon of agglomeration, but it occurred also in controls with normal rabbit-serum. On the other hand, these immune serums caused rapid agglutination of their homologous strains of Leishmania culture forms into enormous masses of distorted organisms with swollen flagella. This specific agglutination cannot be confused with the non-specific agglomeration so frequently observed with various flagellates.

It would appear, therefore, that the flagellates as they exist in the

latex of *A. curassavica*, *A. syriaca*, or *E. brasiliensis*, or in the intestine of *Oncopeltus cingulifer* are immunologically distinct from the 4 strains of human Leishmania. The differences in immune reactions, as well as in cultural properties, between the plant or insect flagellates and human Leishmania may well be an expression of faculties acquired under highly specialized environments. It is also possible that there may exist, among the flagellates in these latex plants, or hemipterans, certain strains which are immunologically and culturally identical with the Leishmania from human sources and are capable of producing the skin infection in man, either directly or after gradual adaptation through insects and lizards, as postulated by Strong.

I wish to thank Dr. Deeks for his courtesy in according me the privilege of working at the Tela Hospital during part of this experimentation, and Dr. Nutter and his associates at the hospital, Dr. Roberts and Dr. Muldoon, for their hospitality during my visit. As already stated, I am indebted to Dr. Hegner and Dr. Iturbe for having given me the benefit of their knowledge, and to Dr. Herbert C. Clark, the Director of the Laboratory of Tela Hospital, for constant coöperation and attention.

EXPLANATION OF PLATES.

PLATE A. Gross appearance of Leishmania cultures grown on media containing either homologous or heterologous immune rabbit serums. The growth is granular or clumpy in homologous, but smooth in heterologous immune serum media. The age of the cultures was 30 days at room temperature (18°C.).

All specimens shown in Figs. 1–8 were stained with Giemsa's solution and magnified x 1250 diameters.

FIG. 1. Culture forms of *Leishmania tropica*, grown 11 days at 18°C. on the leptospira medium.

FIGS. 2, 3, and 4 represent, respectively, culture forms of *L. brasiliensis*, *L. infantum*, and *L. donovani*, grown under the same conditions.

FIG. 5. Herpetomonads from the latex of *Euphorbia brasiliensis* from Tela, Honduras.

FIG. 6. Herpetomonads from the latex of *Asclepias curassavica* (Tela, Honduras). At the lower, right side a human erythrocyte is shown for comparison.

FIG. 7. Herpetomonads from the latex of *Asclepias syriaca* (Long Island, New York).

FIG. 8. Herpetomonads from the intestine of *Oncopeltus cingulifer*.

FIG. 9. A shoot of *Asclepias curassavica* from Tela, Honduras.   Natural size.

FIG. 10. A portion of the stem of the same plant.   Natural size.

FIG. 11. A branch of *Euphorbia brasilicnsis* from Tela, Honduras.   Natural size.

FIG. 12. Dorsal and ventral aspects of male and female of *Oncopeltus cingulifer*. Natural size.
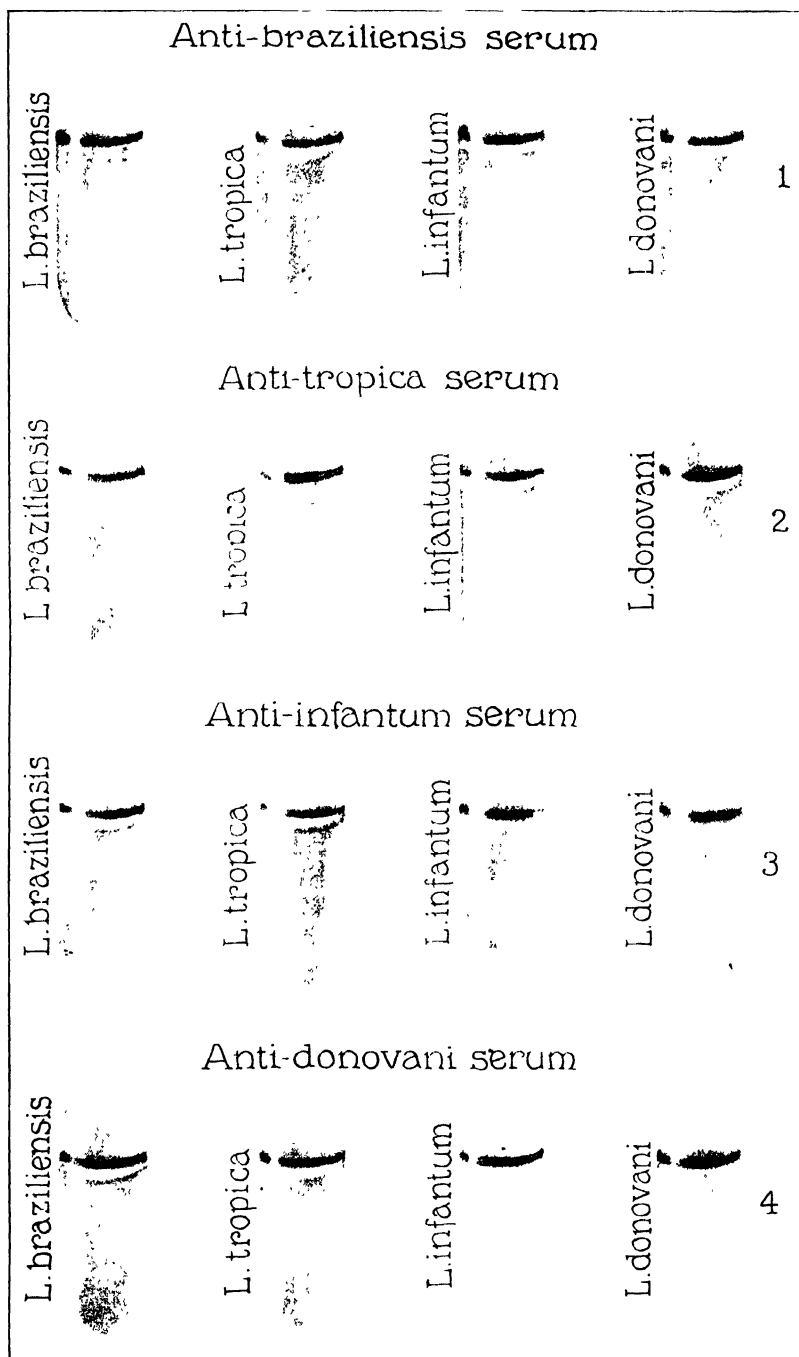
PLATE A. Immunological differentiation of Leishmania in culture.

1　L tropica　　　L. braziliensis　　2

3　L infantum　　　L. donovani　　4
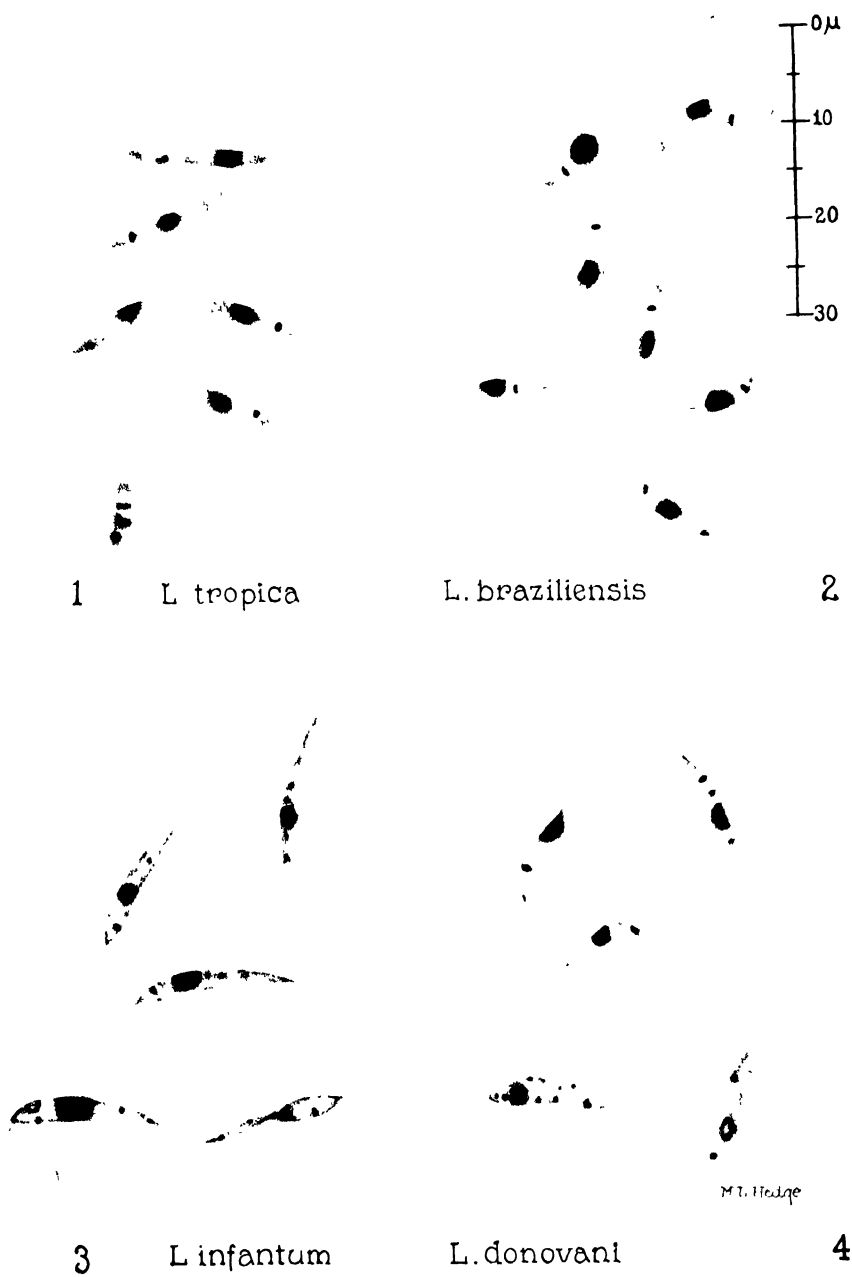
PLATE I. Culture forms of Leishmania.

5 Herpetomonads from
   E. braziliensis

H. from
A. curassavica 6

7 H. from
   A. syriaca

H. from
O. cingulifer 8

PLATE II. Plant and insect herpetomonads.

9
Asclepias
curassavica

♂ ♀

12
Oncopeltus
cingulifer

10

Euphorbia
braziliensis

11

M L Hedge
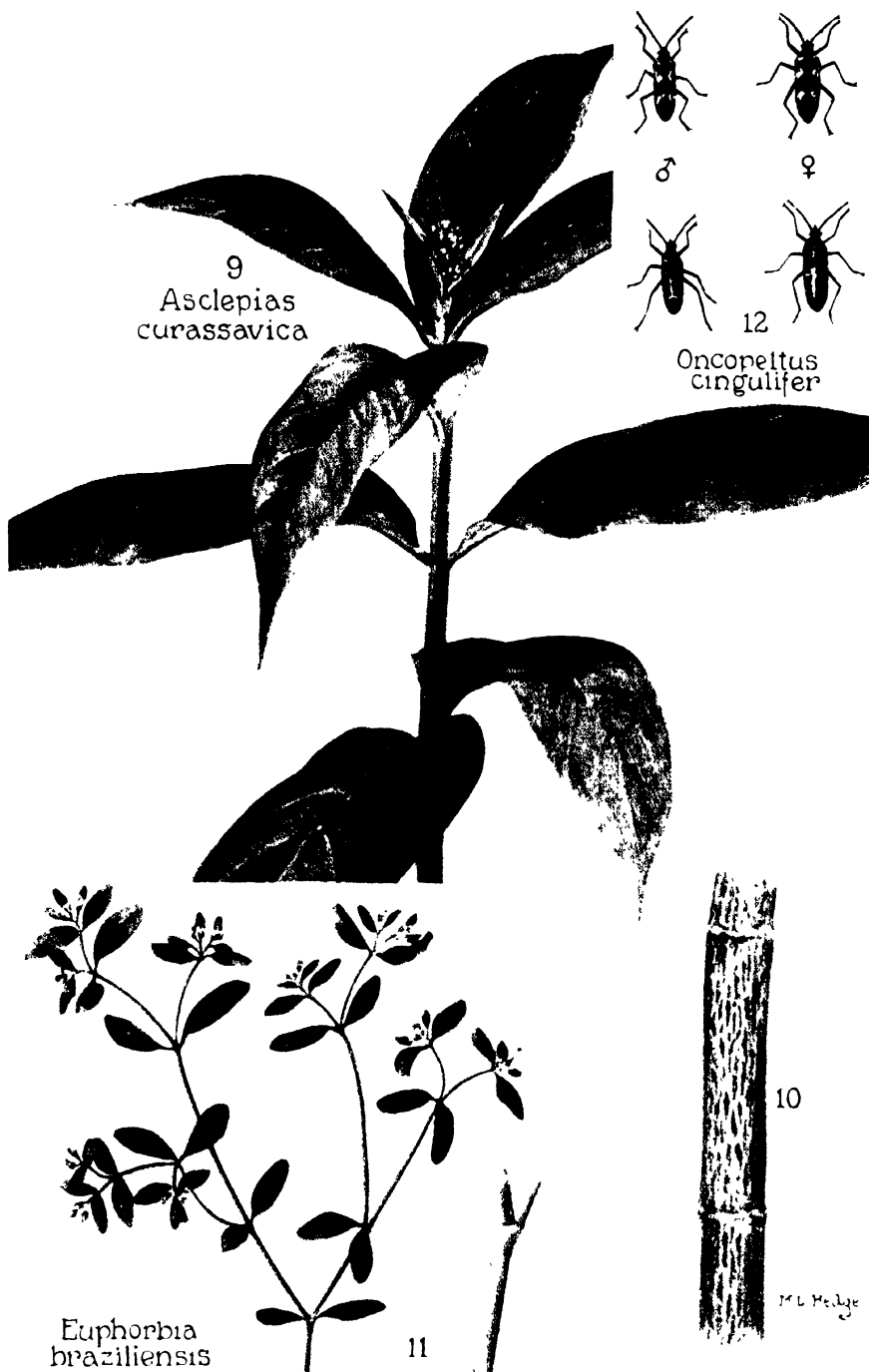
PLATE III. Plant and insect hosts of herpetomonads.

STUDIES OF THE YELLOW FEVER EPIDEMIC IN SALVADOR, C. A., IN 1924.

By HENRY R. MULLER, M.D., and C. BYRON BLAISDELL, M.D.

(Special Members, Field Staff, International Health Board, The Rockefeller Foundation.)

During June, July, August, September, and October of 1924, an epidemic of yellow fever occurred in San Salvador, the capital of the Republic of Salvador, C. A. The diagnosis was made from the clinical symptoms and from the autopsy findings by the local physicians (Segovía, Lardé) and by members of the Rockefeller Commission (Molloy, Bailey, and Hanson). Altogether there were fifty-six recognized and recorded cases of yellow fever of which fifteen were fatal, but the total number of cases is difficult to ascertain exactly. At least five cases occurred in a barracks among soldiers and many in the Casa Presidential, in the hotels and houses within a radius of one or two blocks from the barracks. Although the first recognized case was in a man of German birth who became sick on June 5 and died on June 9, subsequently about fifty cases occurred in natives of Salvador or other Central American countries. It is estimated that about 1,500 foreigners (British, French, Germans, Americans, Syrians, and Chinese) live in San Salvador. That so many natives were affected is partly explained by the fact that many of them were non-immunes from the interior of the republic who had recently come to live in the capital, San Salvador, after having previously resided in the rural districts where yellow fever had not existed.

During the latter part of the epidemic, cases of yellow fever were reported in other parts of the republic, but these outbreaks were relatively small and all occurred in the western part of the country. For instance, in the city of Ahuachapan (eighty miles west of the capital), twenty cases, eight of them fatal, were reported as suspicious of being yellow fever between August 1 and October 16. Also in Chalchuapa (twenty miles from Ahuachapan) between September 17 and October 5, six suspicious cases, with three deaths, were reported.

Unfortunately, we arrived in Salvador a month after the last case of yellow fever was recorded in the country (November 13, 1924), hence we had to content ourselves with a study of such materials as could be gathered from the cases which had occurred earlier. Dr. Juan Segovía, the Director of the Public Health Department of Salvador, Dr. Lardé, the chief of the Anti-Larval Division of the Public Health Department, and Dr. Parada, Chief of the Laboratory of the Public Health Department very kindly turned over to us what could be obtained in the way of histories, autopsy protocols, autopsy tissues, and sera from convalescents and normal individuals. The facilities of the laboratories of the Dirección Géneral de Sanidad also were placed at our disposal. To Dr. M. E. Connor, of the Rockefeller Commission for Yellow Fever Control in Central America, too, we are thankful for constant help and attention.

We directed our attention, first, to a study of the Pfeiffer phenomenon with sera from convalescents. Supplementary to the Pfeiffer reaction in convalescents we carried out similar tests with the sera from groups of normal adults, normal children, and individuals vaccinated against yellow fever. Next we studied the histopathology of yellow fever as it occurred in Salvador.

(I). Pfeiffer Reactions with the Sera of Yellow Fever Convalescents and of Normal Individuals.

Through the kind efforts of Drs. Segovía, Lardé, and Parada, blood was obtained from individuals who had recovered from clinically well defined or clinically suspicious yellow fever during the epidemic of 1924. The sera from these cases were tested against virulent, living cultures of *Leptospira icteroides* in the following manner:

A mixture consisting of 0.5 c.c. serum, 0.5 c.c. pure culture (taken from the surface of a growth on the usual "Noguchi leptospira medium," and 1.5 c.c. of 0.9 per cent. saline was immediately injected into the peritoneal cavity of normal, medium sized guinea-pigs (200 to 300 grm.). At intervals of twenty or thirty minutes, for a period of two hours, samples of the injected liquid were withdrawn from the peritoneal cavity with capillary pipettes and examined by means of a dark-field microscope. Control tests with the saline solution used and with sera from normal individuals, never exposed to yellow fever,

were performed. Since it has been shown (1) that the various strains of *L. icteroides* obtained from Ecuador, Peru, Mexico, and Brazil, are equivalent for this test, they were used indiscriminately. Most of the individual tests were repeated. It has also been repeatedly shown that the lytic substances present in the blood after an attack of yellow fever are specific against *L. icteroides* (1).

As indicated in Table A, of twenty clinically suspected or definite cases of yellow fever occurring in the city of San Salvador during 1924, sixteen give definite positive Pfeiffer reactions, i. e., lysis of the leptospira within a period of two hours. Usually lysis took place in half an hour. One doubtful result was obtained in Case 10 which was clinically a positive case of yellow fever. Of the three negative cases, Case 24 was either not yellow fever or was aborted on the first day by the use of serum. Excluding this case we obtained 84 per cent. positive Pfeiffers in nineteen clinically positive cases of yellow fever.

It has been extremely difficult to get reliable histories from the cases of Chalchuapa and Ahuachapan, and as the specimens of blood were exposed to the rather warm ambient temperature for three days before they reached the laboratory it is possible that the sera may have been altered in their activity. Sera were obtained from five cases and only two gave positive results (Table B). These tests were repeated, with the same result each time. It is quite likely that many of the suspicious cases in these two towns were not yellow fever; for instance, in Case 27 the patient herself gave us a history which was not very suggestive of yellow fever (see Remarks, Table B). In Case 30 also the history states that malaria organisms were found in the patient's blood during her illness, hence this case may have been a severe form of malaria. The sera from these cases gave negative Pfeiffer reactions.

With a view to determining whether certain individuals who had apparently remained well during the epidemic had nevertheless passed through a mild form of the disease without recognizing it, sera were obtained from a group of normal soldiers living in the barracks, where several cases occurred. Of the fifteen individuals (Table C), fourteen gave clear-cut negative results, and one a mild positive result. In this last case (Case 25), however, a vaccination, carried out either in August or September, 1924, may have been responsible for the mild positive reactions. However, negative results were obtained with the

TABLE A.

Pfeiffer Tests with Sera of Yellow Fever Convalescents in Salvador, 1924 (Suspicious and Definite Cases.)

Case number	Name	Date of onset	Date of Pfeiffer test	Number of days between illness and test	Result of Pfeiffer	Remarks
7	E. de G.	2/5/24	12/12/24	224	+	Black vomitus; jaundice; albuminuria.
8	E. G.	9/6/24	12/12/24	186	+	Had much black vomitus; albuminuria late.
9	I. V.	15/6/24	14/ 1/25	213	−	Mild chills and fever, headache, backache, pains in legs, bilious vomiting; sick for 5 days; became jaundiced in last days of illness. Rapid recovery.
10	J. C.	18/6/24	11/12/24	176	±	Is father of Case 8. Less severe than Case 8.
11	M. C.	18/6/24	11/12/24	176	+	Typical case.
12	A. E.	29/7/24	4/12/24	128	+	Typical case with black vomitus; melæna, icterus, albuminuria, and cylindruria; fever 7 days.
13	P. D.	31/7/24	8/12/24	130	+	Icterus; albuminuria.
14	S. M.	12/8/24	2/12/24	112	+	Fever 8 days; oliguria; albuminuria; bradycardia; epistaxis; bleeding from gums.
15	K.	18/8/24	23/12/24	127	+	Diagnosed by Dr. Hanson as a "mild case of yellow fever."
16	S. M.	20/8/24	12/12/24	114	+	No history available; diagnosed as "yellow fever."
17	E. A.	25/8/24	24/ 1/25	152	−	Icterus; oliguria; albuminuria and cylindruria; vomitus spotted with blood; bleeding from gums; menorrhagia.
18	V. G.	28/8/24	2/12/24	96	+	Melæna, icterus; epistaxis; oliguria; albuminuria and cylindruria.
19	J. A. M.	31/8/24	2/12/24	93	+	No history available; diagnosed yellow fever.
20	S.	31/8/24	15/12/24	106	+	Mild case (Dr. Hanson), fever 5 days.
21	A. F.	?/8/24	8/12/24	130?	+	Mild case (Dr. Parada).
22	R. R.	?/8/24	31/12/24	150?	+	No history available, but his physician called it a typical case.
23	C.	4/9/24	15/12/24	102	+	Diagnosed mild case (Dr. Hanson); rapid recovery.

TABLE A—*Concluded.*

Case number	Name	Date of onset	Date of Pfeiffer test	Number of days between illness and test	Result of Pfeiffer	Remarks
24	S.	4/9/24	26/11/24	82	−	Regarded by Dr. Hanson as atypical and questionable; given anti-icteroides serum on first day; improvement in few hours.
25	A. E.	8/9/24	2/12/24	115	+	Mild case, characterized chiefly by albuminuria; oliguria; cylindruria and bradycardia.
26	J. B.	?	8/12/24	?	+	No history available.

TABLE B.

Pfeiffer Tests with Sera of Suspicious Yellow Fever Convalescents from Ahuachapan and Chalchuapa (Salvador), 1924.

Case number	Name	Date of onset	Date of Pfeiffer test	Number of days between illness and test	Result of Pfeiffer	Remarks
27	R. L.	2/9/24	22/12/24	111	−	Suspicious case in Ahuachapan; but no vomiting; no jaundice and no congestion of eyes, according to patient.
28	F. C.	3/10/24	22/12/24	80	+	Suspicious case in Ahuachapan.
29	G. A.	13/10/24	22/12/24	70	+	Suspicious, mild case in Ahuachapan.
30	M. G.	7/ 9/24	23/12/24	107	−	Suspicious case in Chalchuapa; *Plasmodium vivax* and *falciparum* found in blood during illness.
31	C. F.	1/10/24	23/12/24	83	−	Suspicious case in Chalchuapa.

sera of three individuals of this same group who had been vaccinated 130 to 160 days previously (exact date is not available). The tests in this group were all performed between January 12 and 19, 1925, i.e., not more than six or seven months after the first recorded case of

TABLE C.

Pfeiffer Tests with Sera of Normal Soldiers from Barracks Where Yellow Fever Cases Occurred in 1924.

Case number	Name	Result of Pfeiffer	Remarks	Vaccinated against yellow fever
32	A. R. F.	—	Born in Mejicanos (suburb of S. S.); two years in S. S.; in barracks since March, 1924.	No.
33	E. M.	—	Born in Mejicanos (suburb of S. S.); six years in S. S.; in barracks since April, 1924.	No.
34	B. A.	—	Born in San Pedro Perulapán; five years in S. S.; in barracks since March, 1924.	No.
35	C. S.	± weak	Born in Santo Tomás; two years in S. S.	Yes, About 150 days previously.
36	R. C.	—	Born in San Marcos; two years in S. S.	Yes, About 150 days previously.
37	C. G.	—	Born in Chinameca (Dept. San Miguel).	No.
38	C. C.	—	Born in Santa Tecla (suburb of S. S.); seven years in S. S.	No.
39	P. M.	—	Born in San Pedro Perulapán; two years in S. S.	Yes, 130 days previously.
40	P. P. B.	—	Born in San Pedro Masahuat; five years in S. S.	No.
41	A. A.	—	Born in San Miguel; eleven months in S. S.	No.
42	P. E.	—	Born in Tonacatapeque; nine months in S. S.	No.
43	S. J.	—	Born in Jocoro (Dept. Morazán); three years in S. S.	Yes, About 160 days previously.
44	V. LeL.	—	Born in Santiago Texacuango (Dept. S. S.); two years in S. S.; in barracks since Oct., 1923.	No.
45	C. S.	—	Born in San Isidro (Dept. S. S.); two years in S. S.; in barracks since Oct., 1923.	No.
46	T. N.	—	Born in San Antonio Abad (Dept. S. S.); one year in S. S.	No.

S. S. = San Salvador.

epidemic of 1924. From past experience (2 and 3), we would expect the serum still to give a positive reaction if the individual had had the disease within this period, even though in a mild or unrecognized form. As already stated, among fifteen soldiers that were examined, no such clinically unrecognized types of the disease were detected by means of the Pfeiffer reaction.

TABLE D.

Pfeiffer Tests with Sera of Children.

Case number	Name	Age	Result of Pfeiffer	Remarks
47	L. T.	8	—	Born in S. S. and has always lived there.
48	A. M.	8	—	Born in San Miguel; in S. S. since Jan., 1924.
49	C. A. R.	8	—	Born in S. S. and has always lived there.
50	J. C.	8	—	" " "
51	J. O.	8	—	" " "
52	E. E.	8	—	Born in Huizucar, but lives now in S. S.
53	L. G.	8	—	Born in S. S. and has always lived there.
54	J. E.	8	—	" " "
55	J. C. R.	8	—	" " "
56	F. Q.	8	—	" " "
57	A. V.	8	—	" " "
58	V. C.	8	—	" " "
59	E. M.	8	—	" " "
60	A. V.	8	—	" " "
61	H. S.	7	—	" " "
62	V. F.	8	—	" " "
63	A. H.	9	—	" " "
64	A. M.	8	—	Born in Tomanque; in S. S. five years.
65	R. V.	9	—	Born in S. S. and has always lived there.
66	G. M.	8	—	Born in S. S. and lived in suburb of S. S. five years.
67	O. R.	9	—	Born in San Miguel and now lives in S. S.
68	F. S.	6	—	Born in S. S. and has always lived there.

S. S. = San Salvador.

It has been suggested that between epidemic periods the virus of yellow fever may be maintained in children who have the disease in an atypical form, so mild that it has hitherto not been recognized. It has been thought possible that such cases also occur during epidemics. With the hope that the cases might also be detected by the Pfeiffer reaction, sera were obtained from twenty-two children from six to nine years of age (Table D), who had lived in San Salvador during the

epidemic of 1924. All the sera gave absolutely negative results, hence no evidence was obtained in favour of the assumption just mentioned.

Pfeiffer tests were made also on another group of sixteen men (Table E), most of them young adults, natives of Salvador, employed as inspectors for the Anti-Larval Campaign in Salvador. With one exception all the results were negative, and the serum which gave a positive result was from a man (Case 76) who had had yellow fever in Peru in 1920 (diagnosis by Dr. Hanson).

TABLE E.

Pfeiffer Tests with Sera of Sanitary Inspectors.

Case number	Name	Result of Pfeiffer
69	C.	—
70	A.	—
71	S. M.	—
72	E. G.	—
73	C.	—
74	Q.	—
75	B.	—
76	S.*	+
77	J. V. M.	—
78	M.	—
79	G.	—
80	M.	—
81	V.	—
82	L.	—
83	L. C.	—
84	C.	—

* Had yellow fever in Peru, 1920. Diagnosed by Dr. Hanson.

In Table F are listed nine individuals who were vaccinated about three to five months previous to the Pfeiffer test. In no instance was there anything in the history suggestive of yellow fever either before or after vaccination. Of these, three sera gave strongly positive, three weakly positive, and three negative Pfeiffer reactions. These results show that in some vaccinated individuals, even after a period of five months, the serum still has lytic action on *L. icteroides*. The number tested is not large enough to permit more definite conclusions as to the duration of immunity after vaccination.

Among the cases listed in Table G is that of a woman (Case 86) who had yellow fever during an epidemic in Santa Elena, Salvador, in 1900. After an interval of twenty-four years, her serum gave a negative Pfeiffer test. The serum from Case 76 (Table E) still gave a

TABLE F.

Pfeiffer Tests with Sera from Vaccinated, Normal Individuals.

Case number	Name	Number of days between vaccination and test	Result of Pfeiffer test	Remarks
35	C. S.	about 150	+ (weak)	Exact date of vaccination not available.
36	R. C.	about 150	−	" " "
39	P. M.	about 130	−	" " "
43	S. J.	about 160	−	" " "
92	A. S.	152	+ (weak)	Vaccinated, 2 injections, July 22 and 27, 1924.
93	L. S.	152	+ (weak)	" " "
94	A. C. G.	153	+	" " "
95	C. M.	153	+	" " "
96	M. E. C.	91	+	Vaccinated, 1 injection Nov., 1918; 2 injections Feb., 1919; 2 injections Oct., 1924.

TABLE G.

Pfeiffer Tests with Tests with Sera from a Group of Non-Vaccinated, Supposedly Normal Individuals.

Case number	Name	Result of test	Remarks
85	La C.	−	Had malaria when blood was taken for test.
86	S. E.	−	Had yellow fever in Santa Elena, Salvador, 1900.
87	J. M.	+	History suspicious of yellow fever in May, 1924, obtained from individual after the test.
88	M.	−	Born in U. S. A. Has been in Peru several years.
89	J. O.	−	Infant, aged 17 months. No history.
90	C. Q.	−	Malaria in August, 1924.
91	I. G.	+	Born in Chalatenango, Salvador. No history of illnesss.

positive reaction after a lapse of four years. That the duration of the property producing the Pfeiffer phenomenon is variable in different individuals is known, and there are two instances recorded (4) where

positive reactions were obtained after four and four and a half years respectively.

Cases 85 to 91 inclusive (in Table G) were individuals of whose past history nothing was known before the test. They were negative with the exception of Cases 87 and 91 which gave positive Pfeiffer reactions. Case 91 could not be located after the test, hence the history could not be obtained, and there was no serum left with which to repeat the test. In Case 87, however, we obtained the following information from the individual himself:—

Aged 24. In May, 1924, he was taken sick with severe backache, headache, and fever lasting four days. His eyes were congested, but he recalls no vomiting, nor any black or bloody stools. At the end of the attack he thinks he was jaundiced. He was not seen by a doctor. He further stated that an aunt living in the same house had a similar attack at least half a month before he was taken sick.

This history might be interpreted as that of a mild, atypical case of yellow fever, having occurred in San Salvador about one month before the first case that was definitely recognized as yellow fever. In both this case as well as in Case 91 the positive reaction of the serum may have been the result of a yellow fever infection in either this or in a previous epidemic. With the exception of these two cases we were unable to get evidence by means of the Pfeiffer reaction that yellow fever occurs in such a mild form that it is clinically not recognized.

(II). Pathology of Yellow Fever in San Salvador, 1924.

In view of the fact that autopsy material from recent cases of yellow fever appears destined to become increasingly more difficult to obtain, it seems justifiable to present here, in abstract form, histories and autopsy protocols in addition to the descriptions of the histopathology of the newly acquired material, even though the latter is rather scant.

Case 1.—O. F. C. H., aged 29, merchant, German. Onset June 7, 1924. Temperature (buccal) 40°C., marked cephalalgia, rachialgia, discomfort in epigastrium, agitation, bilious vomiting; urine dark, without albumin. June 8: A.M. temperature 39°C., symptoms more pronounced. June 9: Temperature 37.5°C. "Black vomitus" severe. Jaundice, which had appeared after the first twenty-four hours, still present. Almost complete anuria. Delirium in the night. Stools black (melæna). Malaria parasites absent. June 10: Complete anuria (see autopsy); black vomitus abundant; convulsions, fever dropping, pulse almost imperceptible. Coma, death.

Autopsy.—Performed one hour after death by Dr. Zuniga Idiáquez. Skin and sclerae pronouncedly icteric. *Stomach:* Contains large quantity of "coffee ground" liquid. The mucosa has hæmorrhagic spots, and numerous small erosions. *Small intestine:* Contains black material, and both small and large intestine are congested. Some mesenteric lymph nodes are enlarged. *Liver:* Normal in size, yellow (like straw), and friable. *Gall-bladder:* Contains normal bile, but no calculi. *Spleen:* Slightly enlarged, passive congestion. *Kidneys:* Turgid, increased in size, with parts stained yellow. Capsule not adherent. *Bladder:* Contains 30 c.c. markedly albuminous urine. *Pancreas and adrenals:* Normal. *Lungs:* Calcified node in apex of left lung and calcified nodes in hilum of same side. *Heart:* Firm, normal size.

Histological Examination (hæmatoxylin and eosin sections).—The material received by us from this case consisted of pieces of liver and kidney embedded in paraffin. Since no other tissue was available, stains for fat could not be made. *Liver:* Necrosis of cells extends nearly throughout the entire lobule; only small groups of intact cells are on the periphery of lobules and around central veins. The necrotic material is coarsely granular and stains deep red. The other cells, as well as those only slightly necrotic, are vacuolated. A moderate degree of round-cell infiltration exists in the parenchyma and a lymphocytic infiltration in the periportal connective tissue. Mitotic figures were not found, but some nuclei of liver-cells are hyperchromatic and increased in size.

Kidney: There is no necrosis but there are many hyalin- and blood-casts in the tubules. There is a large amount of granular material in the lumen. A number of small hæmorrhages are present. In the capsule of the glomeruli there is an exudate of serum.

Case 2.—J. R. A., aged 17, musician, born in Guadalupe, Department of San Vicente. Became ill on July 13, 1924, and died July 20, in the hospital.

Case 2 was diagnosed as yellow fever, but no history could be obtained. However, an autopsy protocol and pieces of liver and kidney embedded in paraffin were received.

Autopsy.—Autopsy performed by Dr. Lardé, fourteen hours after death. Skin and sclerae markedly icteric. Peritoneum yellow. *Stomach:* Dilated and contains liquid of a dark colour. Mucosa, markedly congested, has ecchymoses and small erosions. These lesions more marked near the cardiac end. *Small intestine:* Contains liquid of same colour as the stomach, and the first portion of the duodenum has lesions similar to those in the stomach. *Spleen:* Weight 210 grm. Firm in consistence and normal in colour. *Adrenals and pancreas:* Normal. *Liver:* Weight 1,602 grm. Yellowish in colour. *Gall-bladder:* Contains 15 c.c. bile, no stones. *Kidneys:* The right weighs 164 grm. and the left 167 grm. Signs of nephritis. *Urinary bladder:* Contains 150 c.c. albuminous urine. *Pericardial sac:* Contains 30 c.c. yellow liquid. *Heart:* Weighs 415 grm. *Lungs:* Congested.

Histological Examination (hæmatoxylin and eosin sections).—*Liver:* Necrosis of the intermediary zone is very extensive, and only very narrow rings of intact cells exist around the central veins and periphery of lobules. Vacuolization

(probably fat) is marked in the preserved cells and those undergoing necrosis in the intermediary zone, but it is much less marked in the cells and the periphery of the lobules and around the central vein.   Mitotic figures are numerous among the preserved liver-cells.   The necrotic masses of cells stain deep red.   An infiltration of round-cells with some polynuclears exists throughout the liver.   In the connective tissue around the portal canals is an infiltration of lymphocytes.

Kidney: The nuclei of the tubules are small and pyknotic, and mitoses are present.   Cell outlines are not visible and many nuclei are absent, indicating necrosis.   The tubules contain hyalin- and red-cell casts.   Small areas of hæmorrhage are present.   There are some lumina that contain large globules, approximately the size of epithelial cells.   These globules are either discrete or conglomerate (some mulberry-shaped) and they stain various shades of blue, generally very dark blue suggesting calcification.   Some are stratified and have concentric rings, and sometimes these blue bodies exist as large granules (figs. 1 and 2).

Case 3.—R. M., aged 21, mechanic, German.   Became sick on July 14 and died on July 20.   No clinical history is available, but an autopsy protocol and pieces of tissue from liver and kidney fixed in Bouin's picroformal and embedded in paraffin were received.

Autopsy.—Performed by Dr. Lardé fourteen hours after death.   Marked icterus of skin and scleræ.   Peritoneum shiny and yellow.   *Stomach:* Dilated, contains a black liquid.   Mucosa is the site of some ecchymoses and numerous small erosions.   *Small intestine:* Contains black liquid.   Duodenum has ecchymoses and erosions.   *Large intestine:* Black fæcal contents.   *Spleen:* Weighs 300 grm.   Dark red.   *Liver:* Weighs 1,110 grm.   Yellowish.   *Gall-bladder:* Contains 15 c.c. bile, but no stones.   *Kidneys:* The right weighs 162 grm. and the left 154 grm.   Colour is dark red.   *Adrenals and pancreas:* Normal.   *Urinary bladder:* Contains 200 c.c. albuminous .urine.   *Pericardial sac:* Contains 30 c.c. yellow liquid.   Some small ecchymoses in the parietal serosa.   *Heart:* Weighs 450 grm.   *Lungs:* Congested and œdematous.

Histological Examination (hæmatoxylin and eosin sections).—*Liver:* Extensive necrosis and vacuolization of almost the entire lobule.   Among the necrotic cells are some cells with preserved nuclei and especially on the periphery of the lobules are these cells more numerous.   Vacuolization is very marked.   There exists a moderate infiltration of round-cells and polynuclears throughout the lobule, and there is a lymphocytic infiltration of the periportal tissue.   Lobules and trabeculæ are not readily made out.   Only small foci of less extensively damaged cells are located on the periphery of lobules and around central veins.   A few mitoses are present.

Kidney: Cloudy swelling of the epithelium of the convoluted tubules exists, and there are nuclei in various stages of karyolysis and karyorrhexis.

Case 4.—V. S., aged 27, born in San Salvador.   Became sick on August 10 with fever, headache, and backache.   Vomitus at first bloody, but later became black.   Seen by physician for first time August 13.   On that day had temperature 38°C., pulse weak, bleeding gums, bloody stools, epistaxis, generalized jaundice, greenish-

yellow papules on lower extremities and on back. Urine scanty. Arrived in hospital August 15, delirious and dyspnœic. Face pale, lips cyanotic; conjunctivæ injected; sclerœ reddish-yellow with some spots of extravasated blood; gums bleeding; extremities cold; stools bloody; no urine obtainable on catheterization; temperature 37.5°C., pulse 52; died at 9 a.m.

Autopsy (time and operator not stated).—Generalized jaundice; ecchymotic spots on cornea (?) of right eye, ecchymotic spots on gums, marked on inferior maxilla; a thin layer of coagulated blood on the labia majora. Peritoneum, strongly icteric; no excess liquid. *Liver:* Weighs 1,260 grm. Consistency is soft, somewhat friable, and has externally an orange colour, with red ecchymoses. On the cut surface lobules cannot be distinguished, and the surface is smooth, yellow, and dotted with hæmorrhages. Gall-bladder and bile-passages are normal. *Stomach:* External surface yellow and hyperæmic. Contains 200 c c. black liquid with bloody detritus in suspension. Many punctate hæmorrhages in the mucosa. *Duodenum:* Contains black material, similar to that in stomach. *Small intestine:* Contains dark, lead-coloured pasty material. The mucosa presents hæmorrhagic suffusions and the serosa numerous ecchymoses. *Large intestine:* Contains melanotic fæcal material. *Spleen:* Weighs 189 grm., and presents a normal appearance. *Kidneys:* The right weighs 147 grm. and the left 152 grm. Consistency is soft and the external surface is red. The cut surface of cortex is studded with punctate hæmorrhages. The medulla is red-wine colour. *Urinary bladder:* Distended with urinous ochre-coloured liquid. The mucosa is hyperæmic. *Adrenals and pancreas:* Normal. *Uterus:* The cervix is ecchymotic and the uterine cavity contains yellow mucus. The tubes are notably congested. *Pleura:* Ecchymotic spots on the visceral layer. *Lungs:* Congested and œdematous. *Heart:* Weighs 295 grm. Myocardium is flaccid and there is hypertrophy of left ventricle. Tricuspid and mitral valves have marginal endocarditic lesions.

Histological Examination.—The material obtained (liver, kidney, heart, and spleen) were in formalin. *Liver:* In the hæmatoxylin and eosin sections the lobules can be made out, but the cords are distinguishable with some difficulty. Liver-cells are preserved only in small groups on the periphery of lobules and around the central vein. The cells between contain numerous fine vacuoles, but actually necrotic cells with lost nuclei are scarce and only occur scattered individually or in minute groups. The cytoplasm stains deep-red with eosin. The cells are swollen and the sinuses nearly obliterated. Among the liver-cells are lymphocytes and polynuclears. Red cells seem to be absent. The fibrous tissue is increased around the portal canals but does not extend completely around the lobules. This connective tissue is markedly infiltrated, chiefly with lymphocytes, but also with a few large mononuclears. Many cells contain very finely granular yellow pigment (bile?). As a rule the nuclei are well preserved, but some are small and pyknotic. Mitotic figures are not found.

In the Scarlet R. sections the liver cords appear to be better preserved. Fatty degeneration is uniformly distributed throughout all the cells of the lobules. The fat is abundant and exists in the form of minute droplets. The epithelial cells of

FIG. 1.
Case 2. "Lime-casts" in kidney. Calcified globules resembling calcified des-
quamated epithelial cells in tubule. Magnification × 370. H. and E. stain.
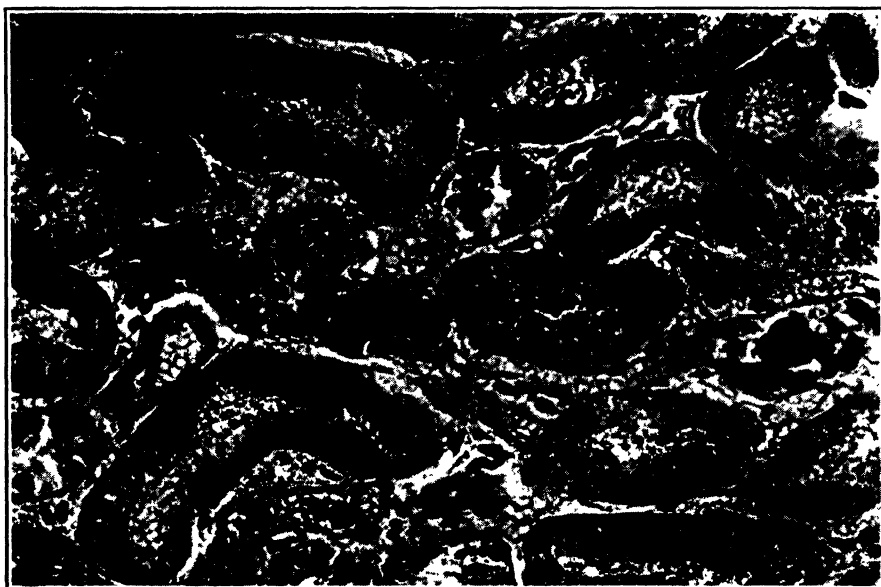
FIG. 2.
Case 2.—"Lime-casts" in kidney. Calcified globules tending to be fused and
forming solid masses. Magnification × 370. H. and E. stain.

the bile-ducts also contain a moderate number of very fine fat-droplets around the nucleus of each cell.

Kidney: In the hæmatoxylin and eosin sections the cells of the convoluted cells are flattened and very granular. The lumen of the tubules is wide and contains abundant loose granular material. The nuclei are generally small and deeply stained, but in many tubes the nuclei are faint or absent. Some tubules contain red blood-cell casts. Many cells of convoluted and collecting tubules contain fine round vacuoles. The capsule of the glomeruli is slightly thickened and there is a mild infiltration of polynuclear and round cells in the glomeruli. The so-called "lime-casts" are not encountered.

In the Scarlet R. sections a fairly marked fatty degeneration of the convoluted and collecting tubules exists, the fat being in the form of minute drops.

Heart: There is fragmentation of the muscle fibres. Small patches of fibres present, fatty degeneration in the Scarlet R. sections.

Spleen: Follicles are small and sinuses somewhat dilated.

Sections of all these tissues impregnated with silver according to the Levaditi method failed to show any leptospira.

Case 5.—J. E. C., aged 39, born in San Martin, came to the capital two months ago. On August 12 he had prolonged chills and high fever, frontal headache, backache, pain in eyes, pains in muscles, joints, and epigastrium. Vomiting was alimentary. Face and conjunctivæ congested. No jaundice or albumin in the urine. August 14: Headache, marked weakness, tiredness. Conjunctivæ slightly congested, scleræ faintly yellow. Vomitus watery and alimentary; stools semi-liquid and yellow. Urine scanty, contained 1.6 grm. albumin per litre. Blood examination showed absence of malaria parasites. August 15: Temperature 38.5°C., pulse 100. Urine (250 c.c. passed in twenty-four hours) contains granular and cellular casts. Persistent "bilious" vomiting. August 16: Temperature dropped to 36°C. Anuria. August 18: Frank icteric tinge of scleræ. Vomitus is "watery." Urine 30 c.c. passed in twenty-four hours. Temperature 36°C., pulse 80. Blood examination: Red blood-cells = 6,000,260; white blood-cells = 7,000; hæmoglobin 95 per cent.; polymorphonuclears 69 per cent.; lymphocytes 25 per cent; large mononuclears 0 per cent.; medium mononuclears 2 per cent.; eosinophils 3 per cent.; transitionals 1 per cent. August 21: Restless, face slightly congested. Temperature 36°C., pulse 104. Urine 100 c.c. in twenty-four hours. August 22: Shows great anxiety; dyspnœic. Vomitus is watery with some spots of blood. Urine 15 c.c. in twenty-four hours. Temperature 36.6°C., pulse filiform, impossible to count. Skin and scleræ jaundiced. Convulsions, death.

Autopsy (time of autopsy and by whom performed not ascertainable).—Jaundice of skin most pronounced on face and front of chest. Peritoneum yellow; no excess amount of liquid. Mesenteric lymph nodes not enlarged. *Stomach:* Dilated and filled with liquid. External surface is orange-coloured, with zones of hyperæmia, and some ecchymoses and blood suffusions. Abundant adherent mucus. *Small intestine:* Contains yellow, semi-liquid material. Few spots of bloody suffusions in mucosa. *Liver:* Weighs 1,500 grm. and is of normal size.

Colour pale yellow, many ecchymoses. Consistency pasty. The cut surfaces are pale red, studded with many yellow islands. *Gall-bladder* and its ducts are normal. *Spleen:* Weighs 109 grm. Normal in colour, but friable in consistency. *Kidneys:* Right weighs 182 grm. and left 189 grm., notably increased in size. The capsules are adherent. Small hæmorrhages present in cortex. *Urinary bladder:* Contains small amount of urine. *Adrenals and pancreas:* Normal. *Heart:* Weighs 343 grm. Musculature hypertrophied, flaccid, and pale. On the edge of the mitral valve are soft, œdematous nodules. *Lungs:* Bilaterally congested.

Histological Examination (pieces of liver, kidney and spleen received in formalin). —*Liver:* The lobules are somewhat less distinctly demarcated than normal, and contain large, fairly well preserved cells around the central vein and on the periphery. The cells between these zones present necrosis and vacuolization corresponding to fat in the Scarlet R. sections. This distribution of altered cells, however, is not uniform in all parts of the section, but there may be irregularly scattered patches of necrotic and vacuolated cells, i.e., "focal" necrosis instead of zonal necrosis. In all these zones of necrosis and fatty degeneration the infiltration of cells, consisting of lymphocytes, large mononuclears, and a few polynuclears, is particularly abundant. Around the portal canals there is an increase of connective tissue and a lymphocytic infiltration.

Kidney: There is moderate dilatation of the convoluted tubules which are filled with granular debris. Some tubules without epithelial cell lining contain deeply blue-staining large globules which may be the desquamated epithelial cells. These clumps, lying in the lumen, resemble the so-called "lime-casts." The globules are either conglomerate and form nearly a solid mass or they are more discrete. Necrosis and mitotic figures are absent. Fatty degeneration, according to the Scarlet R. sections, is relatively slight, being most marked in the collecting tubuies. Hæmorrhages are present in both medulla and cortex.

Spleen: The follicles are small. No pathological changes to be observed. Levaditi sections of these tissues are negative.

Case 6.—H. R., aged 36, farmer, born in San Vicente, has been in San Salvador for past nine months. October 10, at midnight, had severe chill, followed by fever, nausea, and "bilious" vomiting. Had headache, backache, and pains over whole body. He was admitted to hospital October 15. Temperature 39°C., pulse 88 (relative bradycardia). Sub-icteric tinge of skin; scleræ reddish yellow. Conjunctivæ very congested. Abdomen tender to palpation; backache marked: hiccoughs. No malaria parasites found in the blood. Urine contains 1.75 grm. albumin per litre (Esbach) and many granular casts. October 16: Patient is delirious, and general condition is worse. Temperature 36.5° C., pulse weak, 80 beats per minute. Vomitus is watery with black streaks. 100 c.c. urine passed in eighteen hours. October 17: Temperature 36° C., pulse 82. Delirious; persistent hiccoughs. Scleræ frankly yellow. No vomiting; no stools. In afternoon was catheterized and 30 c.c. urine obtained, which contained 12 grm. albumin per litre (Esbach). October 18: Died at 2 a.m.

Autopsy (time and operator not stated).—Skin, conjunctivæ, and scleræ icteric.

Stomach: Greatly dilated, contains 500 c.c. of black liquid. The smooth mucosa has bloody suffusions and punctate hæmorrhages on the lesser curvature and at cardiac and pyloric orifices. *Liver:* Weighs 1,372 grm. Consistency soft and fluctuant, of bright yellow colour and few ecchymoses. On the smooth, yellow, cut surface the lobules cannot be distinguished. *Spleen:* Weighs 140 grm.; appears normal. *Kidneys:* Right weights 120 grm. and left 140 grm. On the cut surface glomeruli appear swollen. Cortex and medulla present small hæmorrhagic spots and streaks. *Heart:* Weighs 304 grm. Consistence is firm and red. *Lungs:* Considerable congestion and œdema, bilateral.

Histological Examination (material received in formalin).—*Liver:* Necrosis and vacuolization of cells of almost the entire lobule and only small groups of cells on periphery of lobule and around central vein are spared. Lobules and trabeculæ can be distinguished with difficulty. The vacuoles, which are fine and numerous in each cell, correspond to the fat stained in the Scarlet R. sections. The groups of necrotic cells correspond in distribution quite closely with the areas of most intense fatty degeneration. The necrotic cells are numerous, and their nuclei are in various stages of degeneration. Polynuclears and mononuclears are found among the necrotic cells. Mitoses are not found.

Kidney: There is marked cloudy swelling with much finely granular material in lumen. Necrosis and mitotic figures are absent. Hyalin and blood-casts are numerous. Hæmorrhages, too, are frequent.

Spleen: There is dilatation of the blood-filled sinuses. Follicles are small. There is much brown pigment, chiefly in macrophages.

Levaditi sections of liver, kidney, and spleen are negative.

The chief centre of interest in the pathology of yellow fever has, for a long time, been the liver. Chiari (5) has again pointed out recently that the two chief lesions of this organ are fatty degeneration and necrosis. The common feature in all the cases he studied was the existence of necrotic cells in the form of disseminated foci. When these are present in large numbers, the intermediate zone is most affected; the presence of necrotic cells most commonly in the intermediate zone is of secondary importance. Constantly among the necrotic cells can be found cells with marked fatty degeneration. The intermediary zonal necrosis is, however, found in numerous septic diseases and cannot be regarded as pathognomonic for yellow fever.

With these statements we fully agree, and the aspect of the livers of our six cases confirms Chiari's findings. Case 5, for instance, presents "focal necrosis" as well as a "zonal necrosis."

With regard to the pathology of the kidney it seems that all grades of acute nephritis are found, ranging from severe necrosis of the epithe-

ium of the convoluted tubules to various grades of "cloudy swelling." Hyalin and blood-casts are quite frequently found in the tubules. In two cases (Nos. 2, 5) so-called "lime-casts" are present. Their formation and presence are perhaps dependent on the type of nephritis existing in any individual case. Since in some instances they resemble calcified desquamated epithelial cells (fig. 1) and are present in tubules where epithelium is absent, or where only a few cells still remain in place, it is possible that they are formed only in those cases of yellow fever in which the type of nephritis is "desquamative;" in bichloride poisoning, for example, "lime-cast" formation is frequent. To the "lime-casts," therefore, we cannot assign any pathognomonic significance. It is possible that in certain epidemics of yellow fever the type of nephritis was such as to favour the formation of lime-casts, but in our present series, unfortunately small, they were not constantly present and were found only twice in six cases. Hoffmann (6), on the other hand, found them in twenty-four out of twenty-eight cases.

Fatty degeneration of the kidney is variable in its intensity and in our series was relatively less pronounced than fatty degeneration of the liver. It is most intense and most constantly present in the collecting tubules, but the convoluted tubules are nearly always affected also.

The spleen in our series presents no lesions. This observation is in agreement with the findings of others.

The negative findings in the Levaditi sections were not surprising when it is noted that the material came from cases 4, 5, and 6, in which death occurred five, ten, and eight days respectively after the onset of disease, i.e.,at a stage when the infectious agent, *L. icteroides*, is known to have disappeared from the body.

SUMMARY.

Among nineteen persons who had recovered from clinically positive cases of yellow fever in San Salvador, in 1924, in sixteen (84 per cent.) the serum gave a positive Pfeiffer test.

Of five persons from the western part of the republic, who had passed through a disease which was clinically suspicious of yellow fever, the sera of two gave positive reactions. The tentative diagnosis of yellow fever in these two cases was thus confirmed by the Pfeiffer tests.

The sera of fifteen soldiers who had lived in San Salvador and in the same barracks where yellow fever had occurred gave negative reactions.

Fifteen normal sanitary inspectors who had no history of yellow fever gave negative Pfeiffer reactions, while the test was positive in the case of one inspector who had had yellow fever in Peru four years previously.

Twenty-two children, from six to eight years of age, who had lived in San Salvador during the epidemic, all gave negative Pfeiffer reactions.

Pfeiffer tests of the sera of vaccinated but otherwise normal individuals showed that in some instances the lytic substances induced by vaccination may still be active against L. icteroides after a lapse of five months.

The pathological findings in six cases of yellow fever are presented. In the liver zonal necrosis was present and, more rarely, focal necrosis, together with marked fatty degeneration. Associated with these changes was a round-cell infiltration and a milder polynuclear infiltration. Mitosis and hæmorrhages were frequently found. In the kidney there was evidence of acute tubular nephritis of varying intensity. Lime casts were present in two of the six cases. Their appearance suggested that they originated from desquamated epithelial cells which had become calcified. The suggestion is offered that lime casts are formed in some but not all types of nephritis associated with yellow fever, occurring especially when desquamation is a feature.

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THE EFFICACY OF TRYPARSAMIDE IN THE CURE OF AFRICAN SLEEPING SICKNESS.

BY CLEMENT C. CHESTERMAN, O.B.E., M.D. (LOND.), D.T.M. & H. (CAMB.).

(From the Baptist Missionary Society Hospital, Yakusu, Haut Congo Belge.)

Ever since the introduction, nearly 20 years ago, by Thomas, of atoxyl into the field of the therapeutics of trypanosomiasis, hopes have been alternately fostered and then relinquished in the attempt to cure certain human infections by the two human trypanosomes, *T. gambiense* and *T. rhodesiense.*

Complete and lasting success has accompanied the use of a variety of drugs in curing the hæmic and lymphatic infections which precede by intervals, varying from a few months to a few years, the migration of the trypanosomes from the blood-vessels and their entrenchment in the tissues themselves, especially those of the central nervous system. Up till the trial by Pearce (1920)[1] of tryparsamide, elaborated by the workers of The Rockefeller Institute for Medical Research, no valid claim had been advanced for anything but a temporary improvement in the signs and symptoms concomitant with this nervous involvement when really established, although the slighter degrees of it had been apparently arrested.

Before definitely making the claim for tryparsamide as a cure for such cases, based on our experience of the drug for the last four years, it may be well to review in brief the value and limitations of the main drugs and treatments in vogue up to the present.

Spontaneous Cure.

There exists a good deal of doubt as to whether this does really occur. One would expect from the well-recognised factors of varying virulence of the infecting strain of trypanosome and the differing degrees of natural and acquired immunity in the host that a fortuitous combination might result occasionally in a natural cure. The latest evidence in support of this contention comes from Todd (1924),[2]

who reports eight cases found infected in 1911 alive and well in 1920, and one of these (no news of others) in 1924. He allows, however, the explanation of extreme chronicity as admissible. In our own series of 650 cases we have not a single instance of survival in good health among the few patients who have escaped, after diagnosis, any treatment whatever, although we are still watching a few cases who are apparently fit after but one or two injections. If some cases of early infection can result in a spontaneous cure, the consensus of opinion is that all cases (with the possible exception of those cited under the next heading) which have been proved to belong to the second class do eventually succumb to their infection.

Treatment by Atoxyl, with or without Tartar Emetic.

There is plenty of evidence to show that really early cases can remain to all intents and purposes cured after a single administration of one of these drugs—i.e., atoxyl, soamin, arsacetin, tartar emetic. Series of cases treated by one to three injections show varying percentages of cures according to dosage and the stage of disease reached, about which there is often no definite information. Even cases, the examination of whose cerebro-spinal fluid had proclaimed them to be in the second stage, have been apparently cured. Blanchard and Laigret (1924)[3] report five such cases alive and well from 6 to 15 years after treatment by atoxyl and arsenobenzols in doses so meagre and few that, in the light of experience with the vast majority of similar cases, such treatment could be disregarded as the primary therapeutic agency.

Opinion is now in favour of repeated injections of 0.015–0.02 g. per kilo body-weight, of atoxyl—i.e., maximum dosage. In a recent epidemic in our own region near Stanleyville about 250 patients have been thus treated in three years, and the percentages of cures and relapses to date are about equal. Treatment was carried out by native boys after diagnosis by the medical man, and consisted in weekly dosage of 1 g. to adults, to a total of 10 to 12 injections. Unfortunately, it was not practicable at the time of diagnosis to determine in all cases by lumbar puncture the exact stage of the disease.

The value of tartar emetic as an adjuvant to this treatment seems not to be very great, for although it is the stand-by of the veterinary surgeon, it is our opinion that its utility in human infections is confined to its use as a substitute for an arsenical should toxic amblyopia develop, or as a temporary trypanosomicidal agent in advanced cases where the onset of severe meningeal reaction is to be feared from immediate adequate dosage with arsenicals. Although tartar emetic alone has been known to cure even a case of infection with *T. rhodesiense*, yet no one would now use it to the exclusion of the arsenicals, and our experience goes to show that if there is any advantage in the combined treatment it is not commensurable with the increased risk and the visits required during the mass treatment of natives during an epidemic.

Treatment by the Arsenobenzols, etc.

The *arsenobenzols* have not been found to give results appreciably better than atoxyl, and their greater cost has prevented trial on a large scale. Now that continental firms are manufacturing "914" of excellent quality and selling it less than one-tenth the price charged by British firms, a further trial of these products might have been advantageous had not there been still better drugs available. The knowledge that they do penetrate the cerebro-spinal fluid suggests that they might have been more efficacious than atoxyl, the penetrability of which is probably less.

Intrathecal Injections of Drugs or Medicated Serum.—This method of attacking the nervous infection was tried by Reichenow in 1914, and retried by Marshall and Vassalo in 1920. Following their advocacy it was tried by many workers, including ourselves, but has now been abandoned as worse than useless.

Bayer "205," or Germanin.—Prolonged sterilisation of the blood and immediate temporary improvement in all patients, which continues in the early cases for many years, has proved the value of this drug. Yet our own series (1924)[4] of 17 cases in the later stages confirms the findings of Van den Branden and Van Hoof (1924)[5] that the value of this drug in nervous infections is less than that of atoxyl. This Dr. F. K. Kleine has admitted to us in correspondence on the subject, and it is evident from the further reports received privately of his series treated in the Congo (1922).[6]

To recapitulate, therefore, there are a variety of drugs which are capable of effecting and maintaining sterilisation of the peripheral circulation for varying periods. Sterilisation is effected most quickly by tartar emetic, less so by atoxyl, and least quickly by Bayer "205," but the reverse order holds good for the maintenance of sterilisation when once effected. This brings out the real value of "Bayer" to the sanitarian concerned with the removal of infectivity. None of these drugs can, however, be relied upon to cure a case which has shown definite nervous involvement.

Cure by Tryparsamide.

Cases treated originally by Pearce and later observed by Van den Branden and Van Hoof, and cases by the last two workers alone at the Laboratory of Leopoldville, afford the very satisfactory figures of 100 per cent. cure of early cases, with unaltered cerebro-spinal fluid. We are able to report a similar success in five such cases, noted for from three and a half months to one year after treatment. Their figures for a series of 35 with altered fluid after an average observation of seven months was 45.7 per cent. still normal, but recent conversation with Dr. Van Hoof shows that the majority of these have remained well up till the present—i.e., an average observation period of nearly three years. These cases were not so far. advanced and were more vigorously treated than our own series of 40 (1923[7] and 1924[8]). Our latest published figure for non-relapse cases in this series was 40.5 per cent., but by the death of one of these (No. 146, Mbalika, the worst case of the whole series) the figure after two and a half to three and a half years falls to 37.8 per cent. It must be remembered, however, that these were all the worst cases one could find, averaging 630 cells per c.mm. in the cerebro-spinal fluid, and were treated by only one course of tryparsamide.

Unfortunately, there are a number of cases which do not react so favourably to tryparsamide, so in claiming that the drug can cure we cannot claim that it is a cure for all cases. This fact is difficult to explain apart from the factors of varying virulence and different degrees of immunity. Personally, I feel that the latter is the most important factor. Pearce believes that the action of tryparsamide,

which is only feebly spirillicide and trypanolytic in vitro, is due largely to its stimulation of natural defences.

That this defensive mechanism is largely dependent on the liver is suggested by work by Sei (1923),[9] who reported that extracts of the liver substance of Bayer "205"-treated guinea-pigs contained a greater concentration of therapeutically active agent in the infections of mice with *T. equiperdum* than those of any other tissue, not excluding the blood. It has also been shown that Bayer "205," though but feebly trypanosomicidal in vitro, becomes at once powerfully so when mixed with liver extract. Mignoli (1924)[10] has shown that the natural trypanosomicidal activity of adult human serum varies with the degree to which the liver cells are functioning. Acute diseases of the liver markedly, and chronic ones in less degree, exert very definite influences. The tolerance of arsenobenzols in syphilis varies also in a similar manner.

Clinical evidence is also forthcoming in support of this view, for all workers agree in the importance of removing hookworm from patients, the deleterious effect of the secondary anæmia produced by ankylostomiasis on the liver being well known. In two cases, complicated by an unrecognised bilharzial infection, we noted bad results during treatment; patients overdosed with atoxyl or tryparsamide often complain of abdominal discomfort and distention; a dirty tongue and highly coloured urine suggest liver trouble. They often put on a lot of flabby flesh, much to their annoyance, also suggestive of defective metabolism. Now that clinical tests for hepatic efficiency suitable for the busy clinician are being evolved, there seems to be a fruitful field for research in order to control treatment in the same way as for syphilis.

Suggestions for Treatment.

Our best control of treatment is the examination of the cerebrospinal fluid for cells and albumin. This latter is simply effected by precipitation of the albumin in 1 or 2 c.cm. by trichloracetic acid 33 per cent., and comparison of the opacity with standardised bacterial emulsions.

It is convenient in treatment to use the common 10 c.cm. syringe, and as the dose of 4 g. should never be exceeded, a 40 per cent. solu-

tion is necessary in order that 4 g. may be given without refilling the syringe. This solution is arrived at accurately by the following simple rule, easily followed by native assistants. *Weigh x g. of tryparsamide and add 2x c.cm. distilled water.* For instance, 10 g. is dissolved in 20 c.cm. water and the resulting solution contracts to 25 c.cm. This is not too strong even for intramuscular injection.

The maximum tolerated dose must be arrived at gradually in advanced cases, but must be maintained whenever possible for at least ten injections, which may be either weekly or fortnightly. Adults will almost certainly relapse, if less than 0.05 g. per kilo body-weight is given, and children can stand almost double this amount—i.e., up to 0.1 g. per kilo, and will indeed tend to relapse without it. Thus a child of 14 weighing about 20 kilos can support as much as 2 g. weekly doses. Substitution of tartar emetic (not Bayer "205") for three weeks or a month, if toxic amblyopia develops, will generally allow resumption of former dosage again. In this connection we may add that we have been using daily injections of sodium thiosulphate 0.45 to 0.9 g. at the first sign of amblyopia, but have no definite evidence worth recording at present as to its value.

A second short course should be given whenever possible six months after the first, the cessation of which is to be guided by progress and the condition of the cerebro-spinal fluid. It must be remembered, however, that the cerebro-spinal fluid will progress towards the normal limits subsequent to the cessation of treatment, if that has been thorough; and an excess of cells, even up to 50 per c.mm. may have entirely disappeared after three months.

If it is desired to combine the treatment with Bayer "205," we consider the most rational method is to alternate the two drugs for the first two or three weeks, giving one of each (not either) per week, and then continuing with tryparsamide. Continuation of Bayer "205" beyond the third injection is of very little use, expensive, and dangerous.

In conclusion, we affirm that tryparsamide can cure African sleeping sickness, although it does not always do so as at present administered, and that it should be the mainstay of our attack on trypanosomiasis. This principle has been adopted by the Belgian Government, which is responsible for the treatment of 50,000 cases annually.

I wish to express my thanks to The Rockefeller Institute for continued supplies of tryparsamide and to Dr. P. H. Manson-Bahr for much kind information and help.

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STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

V. EFFECT OF ELECTROLYTES ON THE RATE OF INACTIVATION OF BACTERIOPHAGE BY ALCOHOL.

BY JACQUES J. BRONFENBRENNER, PH.D., AND CHARLES KORB, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In an earlier paper[1] we have shown that upon the addition of an excess of alcohol to bacteriophage, the activity of the latter is rapidly reduced. This period of rapid inactivation seems roughly to coincide with the process of precipitation occurring when alcohol is added to the lytic filtrate. Further exposure to alcohol affects the residual lytic activity comparatively slowly.[2]

It is known[3-7] that lytic agent is easily adsorbed by various colloids. In our own experience, for instance, mere increase in the concentration of agar in the medium to 1.5 per cent caused loss of over 99 per cent of the activity of lytic filtrate as compared with that observed in the presence of 0.05 per cent of agar.[8,9] It thus seemed possible that the rapid initial inactivation referred to above could be due to the adsorption of lytic agent during precipitation occurring upon the addition of alcohol. In order to see if there exists any relation between the precipitation of the filtrate and the disappearance of its lytic activity, we attempted to vary the intensity of precipitation by controlling the

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concentration of electrolytes, and to follow the change in the rate of inactivation of the lytic filtrate caused thereby.

Effect of Excess of Sodium Chloride on the Rate of Inactivation of Bacteriophage by Alcohol.

The general procedure followed in these experiments consisted in mixing, in a series of tubes, 0.5 cc. of active filtrate (prepared in 0.5 per cent NaCl broth pH = 7.4) with equal amounts of sodium chloride solution of varying concentrations. These mixtures were cooled to 7°C.; to each tube except two were added 10 cc. of cooled 95 per cent alcohol; and the whole series was placed in the ice box. Of the two tubes not receiving alcohol, one served as control of the original activity of the filtrate (A), and the other, receiving salt solution of maximum concentration, but no alcohol, was held to indicate the effect of the salt alone (B) on the activity of the filtrate. After 3 hours at 7°C., the contents of the respective tubes were thoroughly mixed and 0.1 cc. samples removed for titration by serial dilution in broth. The contents of Tubes A and B were diluted with 10 cc. of 0.5 per cent NaCl solution, previous to titration, in order to render the concentration of lytic filtrate in them comparable to that in the remaining tubes of the series.

The results of this titration are indicated in Protocol 1 both in terms of the actual amount of the respective mixtures exhibiting lytic activity, and in terms of the approximate amount of the original lytic filtrate present in each. As in the earlier experiments,[2] readings were taken at the end of 24 and 40 hours, and the final readings were checked by means of transfer to broth seeded with susceptible bacteria.

Effect of Precipitation on the Activity of the Alcoholic Solution of the Lytic Agent.

The results of this experiment show that sodium chloride alone does not affect the activity of the lytic agent during 3 hours exposure, even when its concentration in the filtrate reaches 2.59 M (Protocol 1, B) On the other hand, addition of 10 volumes of alcohol in the presence of 0.085 M NaCl reduces the activity of the filtrate about 100 times (Protocol 1, C). As the concentration of sodium chloride in the filtrate increases the precipitation is more complete and inactivation of the lytic principle is more marked; and when the concentration of sodium chloride in the filtrate reaches 1.79 M, its activity is almost completely destroyed (Protocol 1, G and H) during 3 hours incubation

Protocol 1.

Increase in the Rate of Inactivation of Bacteriophage by Alcohol, Due to Addition of NaCl to the Filtrate.

Tube	A	B	C	D	E	F	G	H
	Control.	Control.	Control.					
Lytic filtrate in 0.5 per cent NaCl broth, cc.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
NaCl solution. Amount, cc.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
NaCl solution. Concentration.	0.085 M	5.1 M	0.085 M	0.35 M	0.85 M	1.7 M	3.5 M	5.1 M
Resulting concentration of NaCl (about), cc.	0.085 M	2.59 M	0.085 M	0.21 M	0.46 M	0.89 M	1.79 M	2.59 M
95 per cent alcohol, cc.	0	0	10	10	10	10	10	10

Contents of tubes thoroughly mixed and placed at 7°C. for 3 hrs.

	A	B	C	D	E	F	G	H
Precipitate.	0	0	+	+	+	++	+++	+++
0.5 per cent NaCl solution, cc.	10	10	0	0	0	0	0	0

Contents of each tube titrated by serial dilution in broth.

Lytic filtrate present in each dilution (approximate). cc.	Actual amount of mixtures transferred to broth. cc.	A 24 hrs.	A 40 hrs.	A Final transfer.	B 24 hrs.	B 40 hrs.	B Final transfer.	C 24 hrs.	C 40 hrs.	C Final transfer.	D 24 hrs.	D 40 hrs.	D Final transfer.	E 24 hrs.	E 40 hrs.	E Final transfer.	F 24 hrs.	F 40 hrs.	F Final transfer.	G 24 hrs.	G 40 hrs.	G Final transfer.	H 24 hrs.	H 40 hrs.	H Final transfer.
5 × 10⁻²	10⁻¹	±	±	+	+	±	+	±	±	+	+	±	+	+	±	+	±	±	+	±	+	+	–	–	–
5 × 10⁻³	10⁻²	+	±	+	+	+	+	±	+	+	±	±	+	+	±	+	++	++	+	–	–	–	–	–	–
5 × 10⁻⁴	10⁻³	±	±	+	±	±	+	±	±	+	±	+	+	±	±	+	++	±	+	–	–	–	–	–	–
5 × 10⁻⁵	10⁻⁴	±	±	+	+	+	+	±	+	+	+	+	+	±	±	+	–	–	–	–	–	–	–	–	–
5 × 10⁻⁶	10⁻⁵	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
5 × 10⁻⁷	10⁻⁶	±	+	+	+	+	+	±	+	+	±	+	+	–	–	–	–	–	–	–	–	–	–	–	–
5 × 10⁻⁸	10⁻⁷	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5 × 10⁻⁹	10⁻⁸	–	–	–	±	±	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5 × 10⁻¹⁰	10⁻⁹	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5 × 10⁻¹¹																									

The amount of precipitate in the respective tubes was recorded as 0 = no precipitate; $+$ = precipitate present; $++$ = moderate precipitate present; $+++$ = copious precipitate present.

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with alcohol at 7°C. The fact that inactivation of the lytic principle in this experiment appears to be of the nature of adsorption secondary to precipitation of the medium, and not analogous to the process of disinfection, as claimed by d'Hérelle, is further suggested by the experiment which follows.

It was seen in earlier experiments[2] that most of the inactivation of lytic agent occurs within a few minutes following the addition of alcohol. Under the conditions of this reaction, apparently not all of the coagulable material is precipitated (Protocol 1 of the present work, C and D), since addition of sodium chloride to the filtrate results in more copious precipitation, with correspondingly greater inactivation of the lytic agent (Protocol 1, F, G, H). It was to be expected, therefore, that if lytic filtrate is mixed with alcohol, and the precipitate is removed, the clear supernatant fluid which is still active[2] will still contain some coagulable material which should come down if sodium chloride is added to it. If inactivation of lytic agent is due to adsorption, this precipitation must be followed by a greater inactivation, as compared with that occurring in the mixture to which no salt was added.

To test the point 1 cc. of cold lytic filtrate (7°C.) was thoroughly mixed with 10 cc. of cold 95 per cent alcohol (7°C.). This mixture was placed on ice, and after 30 minutes the precipitate which was formed was separated by thorough centrifuging at 7°C. (Protocol 2). The supernatant fluid, now containing only a part of its original lytic activity (the greater part being present in the sediment[2]), was placed in two tubes, 5 cc. in each. To one were added 2.5 cc. of cold distilled water (Protocol 2, A), and to the other, 2.5 cc. of cold 5.1 M sodium chloride (Protocol 2, B); both were placed on ice. At intervals, indicated in Protocol 2, portions of each of the mixtures were removed and titrated for lytic activity by serial dilution in broth. The results were read and recorded exactly as in the earlier experiments.[2]

It will be seen from the results recorded in Protocol 2, A that while the activity of the lytic principle in the supernatant fluid (diluted alcohol) remained practically unchanged for hours, inactivation quickly resulted upon addition of sodium chloride to the mixture (Protocol 2, B). The final concentration of sodium chloride did not exceed 1.7 M, and this concentration did not of itself cause inactivation

(Protocol 1, B). However, when the salt solution was added to the clear supernatant fluid, the solution immediately became opalescent, and a fine precipitate soon appeared. Thus it seems that neither the

Protocol 2.

Inactivation of the Alcoholic Solution of Lytic Agent by the Addition of NaCl.

Cold lytic filtrate... 1 cc.
Cold 95 per cent alcohol.................................... 10 cc.

Placed on ice and after 30 min. centrifuged at 7°C.*
Supernatant fluid used further as indicated below:

	A	B
Supernatant fluid (7°C.), cc.........	5	5
Distilled water (7°C.), cc............	2.5	
5.1 M NaCl (7°C.), cc...............		2.5

Placed on ice and samples removed for titration at intervals.

Absolute amount of filtrate present in each tube.	Actual amounts transferred to broth.	1 −			3 −			8 −			1 +			3 +			8 +		
		24 hrs.	40 hrs.	Final transfer.	24 hrs.	40 hrs.	Final transfer.	24 hrs.	40 hrs.	Final transfer.	24 hrs.	40 hrs.	Final transfer.	24 hrs.	40 hrs.	Final transfer.	24 hrs.	40 hrs.	Final transfer.
cc.	cc.																		
7×10^{-3}	10^{-1}	+	±	+	+	−	+	+	+	+	±	−	−	−	−	−	−	−	−
7×10^{-4}	10^{-2}	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
7×10^{-5}	10^{-3}	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
7×10^{-6}	10^{-4}	+	+	+	+	+	+	±	+	+	−	−	−	−	−	−	−	−	−
7×10^{-7}	10^{-5}	+	+	+	±	+	+	±	+	+	−	−	−	−	−	−	−	−	−
7×10^{-8}	10^{-6}	±	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7×10^{-9}	10^{-7}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7×10^{-10}	10^{-8}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

* It is important to keep the temperature as low as possible during centrifuging to avoid an excessive destruction of lytic principle (compare Tables II and III in the second paper of the series[2]).

alcohol nor the salt alone causes inactivation of lytic agent; but when the solution of lytic agent in alcohol is caused to precipitate by the addition of salt, a rapid inactivation of lytic agent is the result.

Effect of Valency of Electrolytes on the Rate of Inactivation of Lytic Agent in the Presence of Alcohol.

If the observed increase in the rate of inactivation of lytic agent by alcohol in the presence of sodium chloride is due, as suggested, to a more complete precipitation of the medium, replacement of sodium chloride by salts with polyvalent ions might be expected to increase further the rate of precipitation and thus to result in a more marked change in the same direction.[10] Or, the same degree of inactivation as that produced with a given concentration of NaCl should be brought about by correspondingly lower concentrations of salts with polyvalent ions.

Since precipitation of colloids may be affected by both anions and cations, depending on their charge,[11,12] we attempted in a preliminary experiment to establish which of the ions of a salt are most active under the conditions of our experiment. Accordingly, we compared the effect of an arbitrarily chosen equimolar solution of three salts: sodium chloride, sodium sulfate, and calcium chloride. The procedure was exactly the same as that illustrated in Protocol 1, except that only one concentration of each salt (0.2 M) was used instead of different concentrations of one salt as before (Protocol 3).

Since sodium chloride and sodium sulfate behaved in the same way, it appears that the valency of the anion was of no significance. On the other hand, the calcium salt showed a considerably greater effect on inactivation than did sodium chloride, although the anion in both cases was the same. It was thus obvious that, in the case of the substances studied at least, the nature of the cation was important in this reaction. In the subsequent study of the effect of valency of ions on the rate of inactivation of the lytic principle, the valency of the cation only was considered.

In the preceding experiments, the duration of exposure of the lytic agent to the combined action of alcohol and salt, before titration, was arbitrarily set at 3 hours. Since one salt might be expected to act more quickly than another, in the following experiment samples of the mixtures were removed from time to time, as indicated in the protocol.

[10] Schulze, H., *J. prakt. Chem.*, 1881–82, xxv, N.S., 431; 1884, xxvii, 320.

[11] Hardy, W. B., *Z. physik. Chem.*, 1900, xxxiii, 385.

[12] Pauli, W., and Flecker, L., *Biochem. Z.*, 1912, xli, 461.

Thus we obtained not only an idea of the total effect after 3 hours, but also curves representing the rates of inactivation of the lytic agent due to each salt. In order to avoid unnecessary repetition, only one such experiment (with $MnCl_2$) will be described in detail. It is under-

Protocol 3.

Comparative Effect of Cations and Anions on Inactivation of Bacteriophage by Alcohol.

	A	B	C	D
	Control.	NaCl	Na₂SO₄	CaCl₂
Lytic filtrate, cc..........	0.5	0.5	0.5	0.5
0.4 M solution of salts, cc...		0.5	0.5	0.5
Distilled water, cc........	0.5			
Resulting concentration of salts.................	0.04 M NaCl	0.24 M NaCl	(0.04 M NaCl) 0.2 M Na₂SO₄	(0.04 M NaCl) 0.2 M CaCl₂

Mixtures were cooled to 7°C.

	A	B	C	D
Cold 95 per cent alcohol, cc......................	10	10	10	10

Placed on ice for 3 hrs. and titrated.

Absolute amount of lytic agent present in each tube.	Actual amounts transferred to broth.	A 24 hrs.	A 40 hrs.	A Final transfer.	B 24 hrs.	B 40 hrs.	B Final transfer.	C 24 hrs.	C 40 hrs.	C Final transfer.	D 24 hrs.	D 40 hrs.	D Final transfer.
cc.	cc.												
5 × 10⁻³	10⁻¹	+	+	+	+	+	+	+	+	+	+	+	+
5 × 10⁻⁴	10⁻²	+	+	+	+	+	+	±	+	+	±	+	+
5 × 10⁻⁵	10⁻³	+	±	+	+	±	+	±	+	+	±	+	+
5 × 10⁻⁶	10⁻⁴	±	+	+	±	+	+	±	+	+	−	−	−
5 × 10⁻⁷	10⁻⁵	±	+	+	±	+	+	−	+	+	−	−	−
5 × 10⁻⁸	10⁻⁶	±	+	+	−	−	−	−	−	−	−	−	−
5 × 10⁻⁹	10⁻⁷	−	−	−	−	−	−·	−	−	−	−	−	−
5 × 10⁻¹⁰	10⁻⁸	−	−	−	−	−	−	−	−	−	−	−	−

stood that in the case of each salt exactly the same procedure was followed throughout, with variation only in the concentration of salts until such concentrations were found for each as caused an inactivation of the lytic filtrate comparable with that produced by alcohol in the presence of 1.7 M sodium chloride.

Protocol 4.

Comparative Effect of Different Concentrations of MnCl₂ on the Rate of Inactivation of the Lytic Agent in the Presence of Alcohol.

	NaCl	MnCl₂	MnCl₂	MnCl₂
Salt used				
Laudman Shiga filtrate, cc	1	1	1	1
Concentration of salt used	3.4 M	1 M	0.3 M	0.1 M
Amount of salt solution used, cc	1	1	1	1
Approximate resulting concentration of salt	1.7 M	0.5 M	0.15 M	0.05 M
Amount of the above mixture (A), cc	1 (control) — 1	1 (control) — 1	1 (control) — 1	1 (control) — 1
Cold water, cc	10	10	10	10
Cold alcohol, cc	10	10	10	10

Duration of exposure at 7°C:

Amount of original filtrate in each tube, cc.	Amount of mixtures used, cc.	NaCl A 3 hrs	NaCl B 5 min	NaCl B 15 min	NaCl B 1 hr	NaCl B 3 hrs	MnCl₂ A 3 hrs	MnCl₂ B 5 min	MnCl₂ B 15 min	MnCl₂ B 1 hr	MnCl₂ B 3 hrs	MnCl₂ A 3 hrs	MnCl₂ B 5 min	MnCl₂ B 15 min	MnCl₂ B 1 hr	MnCl₂ B 3 hrs	MnCl₂ A 3 hrs	MnCl₂ B 5 min	MnCl₂ B 15 min	MnCl₂ B 1 hr	MnCl₂ B 3 hrs
5 × 10⁻³	1 × 10⁻¹	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5 × 10⁻⁴	1 × 10⁻²	+	+	+	+	−	+	+	−	−	−	+	+	+	−	−	+	+	+	+	+
5 × 10⁻⁵	1 × 10⁻³	+	+	+	−	−	+	+	−	−	−	+	+	−	−	−	+	+	+	+	+
5 × 10⁻⁶	1 × 10⁻⁴	+	+	−	−	−	+	−	−	−	−	+	+	−	−	−	+	+	+	+	+
5 × 10⁻⁷	1 × 10⁻⁵	+	+	−	−	−	+	−	−	−	−	+	+	−	−	−	+	+	+	+	−
5 × 10⁻⁸	1 × 10⁻⁶	+	+	−	−	−	+	+	−	−	−	+	+	−	−	−	+	+	+	−	−
5 × 10⁻⁹	1 × 10⁻⁷	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−	+	+	−	−	−
5 × 10⁻¹⁰	1 × 10⁻⁸	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−
5 × 10⁻¹¹	1 × 10⁻⁹	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

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In each case, to several 1 cc. portions of Laudman Shiga lytic filtrate (prepared in unbuffered broth), was added 1 cc. of varying concentrations of salts (in this case $MnCl_2$); after thorough mixing and cooling to 7°C., two portions of 1 cc. each of this Mixture A were removed. To one of these were added, in each case, 10 cc. of cold alcohol, to the other 10 cc. of cold water, and both sets of tubes were placed in the ice box. At intervals indicated in Protocol 4, B, the contents of the tubes containing alcohol were thoroughly mixed, after which samples were removed for immediate titration by serial dilution in broth, and the tubes were replaced in the ice box. After the last titration (3 hours after the beginning of the experiment), the second tube of each series which received no alcohol was also titrated to show what effect, if any, the salt alone had on the activity of the filtrate during the entire period of the experiment. The results of
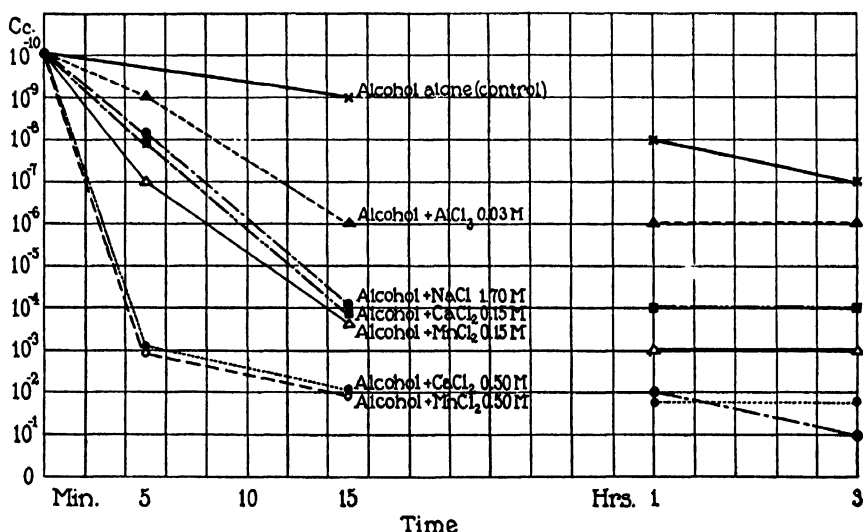
CHART 1. Effect of electrolytes on inactivation of the lytic filtrate by alcohol.

these titrations were read exactly as before (Protocol 1), that is after 24 hours, 40 hours, and after a subsequent control transfer to fresh broth,—but for the sake of simplicity only the final reading has been recorded in Protocol 4.

As can be seen from Protocol 4, the rate of inactivation of lytic agent in the presence of 1.7 M NaCl was most closely approached in the tube containing 0.15 M $MnCl_2$. On the composite Chart 1 are plotted the results of several such experiments. This chart shows again that immediately after the addition of alcohol there occurred a rapid diminution in the activity of the filtrate. This inactivation proceeded

at a fairly constant rate for about 15 minutes. Later, the curves show a distinct tendency to flatten, possibly coincident with the completion of precipitation. This chart shows further that approximately the same rate of inactivation resulted in the presence of 1.7 M sodium chloride as in that of 0.15 M calcium chloride or 0.15 M manganese chloride. In the case of aluminum chloride or lanthanum chloride, a concentration of a little over 0.03 M would accomplish approximately the same inactivation.[13]

Thus, in general, salts with polyvalent cations increase the rate of inactivation of lytic filtrate in the presence of alcohol much more than does sodium chloride under similar conditions. The greater the valency of the cation, the greater is the rate of inactivation. Although there were some minor deviations, which will be discussed in detail in one of the later papers of this series, the nature of cation as apart from its valency influenced the respective rates of inactivation of the lytic agent but little; in many instances no measurable difference could be detected. Thus, for instance, the increase in the rate of inactivation of the lytic agent resulting from the increase in the concentration of calcium chloride from 0.15 M to 0.5 M was duplicated by a corresponding change in the concentration of manganese chloride (Chart 1). Similar parallelism was observed when the concentrations of $AlCl_3$ and $LaCl_3$ were varied.

Effect of Partial Removal of Salts by Dialysis on the Rate of Inactivation of Bacteriophage by Alcohol.

The results of the preceding experiments consistently indicated an intimate relation between the rate of precipitation of lytic filtrate and the loss of its activity resulting from the addition of alcohol. It has been shown that addition of electrolytes increases this inactivation, and that the rate of increase depends on the valency of the cation. Since the lytic filtrate, consisting of beef infusion broth, already contains various salts in addition to 0.5 per cent sodium chloride added to it in the process of preparation, it was thought that perhaps even the initial inactivation of bacteriophage by alcohol[2] (prior to the addition

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[13] The two latter salts could not have been used in concentrations higher than 0.03 M on account of the fact that the change in hydrogen ion concentration in the solution attained thereby was detrimental to the activity of the lytic agent.

Protocol 5.

Activity of Dialyzed Lytic Filtrate.

Actual amount of fluid having lytic activity. cc.	A. Original lytic filtrate. (Lytic power)	B. Outer fluid after first 24 hrs. of dialysis. (Lytic power)	Outer fluid after second 24 hrs. of dialysis. (Lytic power)	Outer fluid after third 24 hrs. of dialysis. (Lytic power)	Outer fluid after fourth 24 hrs. of dialysis. (Lytic power)	Outer fluid after fifth 24 hrs. of dialysis. (Lytic power)	C. Dialyzed lytic filtrate (after 5 days of dialysis). (Lytic power)	D. Original lytic filtrate kept on ice for 7 days. (Lytic power)
1×10^{-1}	+	+	+	+	+	+	+	+
1×10^{-2}	+	+	+	+	+	+	+	+
1×10^{-3}	+	+	+	+	+	+	+	+
1×10^{-4}	+	+	+	+	+	+	+	+
1×10^{-5}	+	+	+	+	+	+	+	+
1×10^{-6}	+	+	+	+	−	+	+	+
1×10^{-7}	+	−	−	−	−	−	+	+
1×10^{-8}	+	−	−	−	−	−	+	+
1×10^{-9}	+	−	−	−	−	−	+	+
1×10^{-10}	−	−	−	−	−	−	−	−
1×10^{-11}	−	−	−	−	−	−	−	−
Chloride.		Present.	Present.	Present.	Present.	Tr.	Present.	

of electrolytes) might be due largely to this presence of salts in it. We therefore attempted to reduce the salt content of the lytic filtrate by dialysis, and to compare the rate of inactivation (by alcohol) of the resulting lytic filtrate with that of the original filtrate.

For this purpose, 50 cc. of ordinary beef infusion broth containing 1 per cent of peptone and 0.5 per cent of sodium chloride, and adjusted to neutral reaction, were seeded with 0.1 cc. of a suspension of young *B. dysenteriæ* Shiga (grown for 18 hours on an agar slant) containing 100,000,000 microorganisms, and incubated at 37°C. for 1 hour. At this time, 0.1 cc. of a dilution of Laudman Shiga lytic principle 1:10,000 in broth was added to the culture. The latter was further incubated for 40 hours, filtered, and the filtrate placed on ice after having been tested for sterility and for its lytic action (Protocol 5, A). 2 days later, when the tests showed the filtrate to be sterile, a 5 cc. portion of it was removed and placed in a sterile collodion thimble surrounded by 200 cc. of sterile distilled water. Both the dialyzing thimble and the remaining filtrate were replaced on ice. The next day, as well as on each succeeding one, the liquid surrounding the dialyzing thimble was removed, tested for the presence of chlorides, and titrated for its lytic activity (Protocol 5, B). At the same time, 200 cc. of fresh sterile distilled water were introduced in place of the liquid removed, and the flask was returned to the ice chest. After 5 days of dialysis against distilled water, the contents of the collodion bag were likewise subjected to the qualitative test for chlorides, and to titration for their lytic power (Protocol 5, C) as well as for sterility. At the same time, a portion of the original filtrate was also titrated for comparison (Protocol 5, D). It will be observed that the lytic filtrate remained sterile; that it was not entirely freed of chlorides by the end of 5 days of dialysis (Protocol 5, C); also, together with chlorides, a certain portion of lytic agent dialyzed and thus was removed daily (Protocol 5, B). This amount was so small, however, that it was not detected when the contents of the bag were titrated after the 5 days of dialysis (compare C and D in Protocol 5) by the usual method of tenfold dilution.

Having thus established the fact that lytic filtrate, partially dialyzed against distilled water, retained practically its full activity in so far as the method of titration permitted estimation, we next proceeded to inquire into the effect of alcohol upon it.

Two portions of 3 cc. each of dialyzed lytic filtrate were placed in tubes;[14] to one of them (Protocol 6, A) was added 0.05 cc. of water, and to the other 0.05 cc. of 30 per cent sodium chloride solution (Protocol 6, B). At the same time, a 5 cc.

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[14] The filtrate, during dialysis, became diluted through osmosis, so that instead of the original volume of liquid placed in the bag (5 cc.), the bag contained by the end of the 5th day approximately 11 cc. of fluid.

Protocol 6.

Effect of Partial Dialysis on Resistance of Bacteriophage to Alcohol.

	A	B	C
Dialyzed filtrate (diluted 5:11 during dialysis), cc.........	3	3	3
Original filtrate (diluted 5:11), cc......			
0.5 per cent NaCl solution, cc......	0.05		0.05
Distilled water, cc.......			
30 per cent NaCl solution, cc........		0.05	
Resulting concentration of NaCl........	Definite traces.	About 0.5 per cent.	About 0.5 per cent.

Placed on ice for 3 hrs.

	A	B	C
From the above mixtures (cold), cc........	1	1	1
95 per cent alcohol (cold), cc........	10	10	10

Placed on ice and titrated at intervals.

Amount of mixtures used. $cc.$	Amount of original filtrate in each tube. $cc.$	A 15 min.	A 1 hr.	A 3 hrs.	A 24 hrs.	A 48 hrs.	B 15 min.	B 1 hr.	B 3 hrs.	B 24 hrs.	B 48 hrs.	C 15 min.	C 1 hr.	C 3 hrs.	C 24 hrs.	C 48 hrs.
5×10^{-2}	1×10^{-0}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5×10^{-3}	1×10^{-1}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5×10^{-4}	1×10^{-2}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5×10^{-5}	1×10^{-3}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
5×10^{-6}	1×10^{-4}	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−
5×10^{-7}	1×10^{-5}	+	+	+	+	−	+	+	+	−	−	+	+	+	−	−
5×10^{-8}	1×10^{-6}	+	+	+	+	−	+	+	−	−	−	+	+	−	−	−
5×10^{-9}	1×10^{-7}	+	+	+	−	−	+	−	−	−	−	+	−	−	−	−
5×10^{-10}	1×10^{-8}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

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portion of the original filtrate (not subjected to dialysis) was diluted to 11 cc. with a 0.5 per cent NaCl solution (in order to duplicate the dilution of the dialyzed portion), 3 cc. of the resulting mixture were placed in a third tube (Protocol 6, C), and to this was further added 0.05 cc. of 0.5 per cent NaCl solution.   The contents of the tubes were thoroughly mixed, and now 1 cc. portions of each were put into cooled tubes and placed on ice.   To the contents of each tube, after cooling, were added 10 cc. of cold 95 per cent alcohol, the tubes were placed on ice, and after thorough mixing of the contents samples were titrated at intervals, as indicated in Protocol 6.

The findings of this experiment indicate that removal of a part of the salts from the lytic filtrate resulted in its greater resistance to alcohol (Protocol 6, A), and that replacement of sodium chloride in the dialysate to approximately the same concentration as that in the original broth results in the increase in its sensitiveness to alcohol (Protocol 6, B).   That in the latter case (Protocol 6, B) the rate of destruction does not quite attain the level observed in the control (Protocol 6, C) is possibly due to the fact that, during dialysis, in addition to sodium chloride some other salts were also removed from the filtrate.

DISCUSSION.

The results of the experiments reported in this paper suggest, in our opinion, that the inactivation of the lytic principle by alcohol is not due to the virucidal action of the latter, but to the adsorption of dissolved lytic substance on the surface of the precipitate occurring when alcohol is added to the lytic filtrate.   However, our results lack the character of indisputable proof.   Thus, for instance, those in favor of the parasitic nature of bacteriophage might take the view that the addition of electrolytes in our experiments increased the disinfecting action of alcohol by forcing it out of the water, and concentrating it in the lipoid constituents of the supposed cell membrane of the *"bacteriophagum intestinale."*

We shall consider this possibility in later experiments.   For the present, however, such an explanation does not seem satisfactory. In the first place, such analogy with the effect of sodium chloride on the bactericidal effect of phenol[15] does not suffice to explain the effect of valency of salts as observed in our experiments.   Furthermore, if the

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[15] Spiro, K., and Bruns, H., *Arch. exp. Path. u. Pharmakol.*, 1898, xli, 355.

inactivation of lytic agent were due in the final analysis to direct toxic action of alcohol, one would expect the rate of the reaction to be expressed by a straight line, as is the case with the rate of lysis of erythrocytes by alkalies,[16,17] for example, or with the rate of destruction of bacterial spores by mercuric chloride,[18] as calculated by Morawitz.[19] As a matter of fact, we have seen that for the first few minutes the inactivation of lytic agent by alcohol proceeds at a rate consistent with the formula proposed by Ostwald for disinfection, but soon the curves flatten out, in spite of the presence of an excess of both bacteriophage and alcohol in the solution. This break in the rate of inactivation of the lytic agent suggests that the reaction which takes place is not due to the alcohol directly, but to some other factor set into action by the addition of alcohol, but ceasing to operate after the first few minutes. We believe this factor is the formation of a fine precipitate with the concomitant adsorption of the lytic agent.

The adherents of the vitalistic theory of the bacteriophage action might suggest that the filtrate representing, according to their views, a suspension of *"bacteriophagum intestinale,"* by analogy with bacteria, might be composed of variants possessing varying degrees of susceptibility to the destructive effects of alcohol. Thus, if the majority of individuals were highly susceptible, and the remainder highly resistant to toxic action, the result would be, in general, similar to that which we observed; namely, the majority of the hypothetical organisms would be destroyed soon after the addition of alcohol, and the filtrate would show a marked and rapid fall in its activity. The remainder of the organisms would be destroyed at a different and much slower rate and consequently the curve expressing the rate of inactivation would show a break.

That such a view is untenable follows from our earlier findings that lytic filtrates are uniform in their susceptibility to the action of alcohol, and contain no fraction possessing a higher degree of resistance.[2]

Still another objection might be raised against our interpretation of

[16] Gros, O., *Biochem. Z.,* 1910, xxix, 350.

[17] Stadler, E., and Kleemann, H., *Biochem. Z.,* 1911, xxxvi, 301, 321.

[18] Krönig, B., and Paul, T., *Z. Hyg. u. Infectionskrankh.,* 1897, xxv, 1.

[19] Morawitz, H., *Kolloidchem. Beihefte,* 1909–10, i, 301.

the results here presented. Lytic agents have been adsorbed on a number of organic and inorganic colloids. Usually they are not permanently inactivated thereby. In the case of the adsorption of the lytic agent by the precipitate resulting from precipitation of the lytic filtrate by alcohol, however, there occurs a marked irreversible reduction in its activity. This destruction, in our opinion, finds its analogy in the similar effect of alcohol upon enzymes and toxins, and is due to denaturation of the protein vehicle on which lytic agent is distributed in a very thin (monomolecular ?) layer.

Somewhat similar observations may be quoted with respect to the destruction of bacteriophage by heat. It has been found by a number of investigators that bacteriophage is relatively heat-resistant, and requires at least ½ hour's exposure at 70–90°C. for complete destruction. However, when it is adsorbed on the surface of bacteria, it is destroyed at 56°C.[20]

Another analogy is seen in our experiments[21] in which bacteriophage is completely inactivated by acetone if electrolytes are added to the filtrate before precipitation, whereas without the addition of electrolytes bacteriophage is found practically unaffected by the acetone.

SUMMARY AND CONCLUSIONS.

Addition of neutral salts to the lytic filtrate results in an increased rate of inactivation of the latter when alcohol is added to it. This effect of salts is the more marked the higher the valency of the cation. Conversely, removal by dialysis of salts originally present in the lytic filtrate tends to render lytic agent less sensitive to alcohol. Restitution of the original salt content to the dialyzed filtrate tends to bring the sensitiveness to alcohol in the dialyzed filtrate to the level of the non-dialyzed control.

It appears, therefore, that inactivation of the lytic agent by alcohol depends directly on the rate of precipitation of the coagulable constituents of the medium, and is not the result of a direct toxic action of alcohol on *"bacteriophagum intestinale."* Considered in association with our earlier findings, these results speak in favor of the chemical nature of the agent of transmissible lysis.

[20] Seiffert, W., *Med. Klin.*, 1922, xviii, 997, 1093, 1121.
[21] Bronfenbrenner, J. J., *Proc. Soc. Exp. Biol. and Med.*, 1925 (in press).

THYMECTOMY IN THE RABBIT.

BY CHESTER M. VAN ALLEN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 1.

Despite extensive investigations, the effect of surgical removal of the thymus in animals is still somewhat uncertain. This uncertainty is due, in part, to the difficulty that has been experienced in distinguishing between effects of thymoprivia and effects that might be attributable to other causes, especially complications arising during or subsequent to operation, postoperative care of animals, and the use of methods that give varying degrees of success with respect to operative injury and complete removal of the gland.

In the course of studies that were being carried out in these laboratories, it became desirable to compare the behavior of thymectomized rabbits with that of normal rabbits and rabbits from which other glands had been partially or completely removed. This necessitated an investigation of the operation of thymectomy itself and the immediate or early results of removal of the thymus. It was necessary, first, to devise an operation that could be depended upon for complete removal of the thymus with a minimum loss of animals, and, second, to determine whether the thymus could be removed from rabbits of certain ages without producing any serious impairment of health, within specified periods of time, which would interfere with their use in other experiments. A preliminary report on one phase of this work has been published (1). The object of this paper is to report the early or immediate results of thymectomy, obtained by a method that was developed for use in connection with the experiments referred to above.

Methods.

As the success of any experimental study of thymoprivia hinges upon the operation, it will be described in detail.

Reviews of the various methods which have been recommended for removal of the thymus in animals may be found in the papers by Asher (2), Matti (3), and Park and McClure (4). The two principal types of operation are represented by the closed method of Friedleben (5), which was introduced in 1858, and the open or transthoracic operation suggested by Basch (6–9) in 1902. In the method of Friedleben, an incision is made at the base of the neck, exposing the upper poles of the thymus; these are seized with blunt forceps′and the gland withdrawn from the chest without attempt at hemostasis. At best, complete ablation is not certain by this approach and mortality is high.

In the transthoracic operation of Basch, the mediastinum is exposed anteriorly by splitting the sternum and the gland is removed by dissection. Unfortunately, Basch left no detailed description of his technique. The method has been modified by MacLennan (10) for use in rabbits, by Ranzi and Tandler (11), who introduced the Sauerbruch chamber, and by Klose and Vogt (12–15) and Park and McClure (4) in using positive pressure anesthesia.

Method Used.—The method of operation employed by us is essentially that of Basch, adapted to the rabbit. All operations were done under ether anesthesia; at first, we used positive pressure apparatus for the prevention of pulmonary collapse but found that this measure might be omitted.

Regional Anatomy.—The thymus of the rabbit is a bilobed organ, flat and roughly triangular in shape. It lies almost entirely within the chest and occupies the anterior mediastinum, the lobes uniting along the midline. The base of the gland usually lies at the level of the fourth rib; the apex reaches through the upper thoracic strait and in the form of two separate strands follows the carotid arteries upwards a variable distance into the neck; the lateral margins of the lobes lie in contact with the pleuræ. Ventrally the thymus is confronted by the chest wall, from which it is separated only by an aponeurosis containing muscle fibres, the substernal extension of the pretracheal muscle layer. ˙Behind the thymus at its lower third is situated the base of the heart covered by the pericardium, at its middle third the aortic arch, and behind its apex is located the great carotid trunk which bifurcates at this point to form the two common carotid arteries. The blood supply of each lobe is received at the lateral margin at the level of the first rib, the artery branching usually from the corresponding internal mammary, and the vein contributing to the subclavian vein (see Fig. 2). Occasionally the gland receives a branch underneath from the carotid artery. The intrathoracic portion of the thymus is encased in loose, cellular connective tissue, which can be made to separate easily, but in the neck the gland processes are held firmly attached to the adjacent vessels. Important nerve structures, as for instance the vagus and phrenic, lie well behind and lateral to the thymus and are not encountered in the operative field. There is one other structure to be noted. This is a vein of good size that runs transversely in a position just above the upper end of the sternum and superficial to the pretracheal muscle layer and connects the jugular veins.

Operation.—On the day of operation, the usual feeding should be omitted. The

fur over the neck and chest is clipped and the skin is shaved from the larynx to the ensiform, dried, and treated with alcohol.

Ether is given by the ordinary open cone method. The animal is draped with two towels wrung out of 1:10,000 bichloride solution, the margins meeting in the midline over the field of operation and widely overlapping elsewhere. The operator should work from the left side.

The incision is made in the midline from the level of the fifth rib to the thyroid cartilage, and the skin is excluded from the operative field by clamping its edges to the margins of the draping towels. The incision is then continued into the left pectoral muscle, following the margin of the sternum from the fifth rib upwards to the base of the neck, where the attachment of the left sternomastoid muscle to the sternum is also divided. This exposes the costal cartilages at their sternal extremities together with the adjacent intercostal muscles. The soft tissues of the neck are entered along the midline by incising between thumb forceps, exposing the trachea from the sternum to the larynx. During this procedure the transverse vein at the base of the neck is sought for, double clamped, and divided. The thorax is then opened as follows: The upper end of the sternum is elevated with thumb forceps and the tissues are separated from its under surface by blunt dissection for a distance of about 1 cm. This permits the blade of a pair of scissors to be inserted beneath the first left costal cartilage close to the sternum and this structure divided without injury to the underlying parts. Elevation of the sternum is maintained while the second, third, and fourth ribs and adjacent intercostal muscles are cut at the left sternal margin. Bleeding from the intercostal vessels is controlled with clamps. The extensive opening into the chest thus obtained is prevented from diastasis by the presence of the substernal aponeurosis, and this must be divided with scissors. The incision may then be widely opened and the anterior mediastinum brought to view (see Fig. 1).

Before proceeding further, preparations are made for the closure of the chest. For this purpose a heavy silk suture is passed about the tip of each cut rib and tied as illustrated in Fig. 1. Care should be taken to avoid piercing the internal mammary vessels. Sutures are also passed and tied around the sternum just above each rib stump. One end of each suture is left long after tying, to form a leash of four threads attached to the bony framework on each side of the thoracic incision. These serve temporarily as retractors.

Removal of the thymus is now begun, commencing at the apex. One of the tongue-like processes of the gland in the neck is grasped with thumb forceps and separated by blunt dissection from the corresponding carotid artery as far downwards as the aortic arch. The other apical tag is freed in the same way (see Fig. 2). This dissection is somewhat difficult because of firm attachments and must be done with care, and one should watch for the glandular vessels that occasionally spring from the carotids. Then the base of the thymus is located in the pericardial fat; its edge is picked up in the midline, and by drawing upwards upon it the gland is peeled away readily, and without dissection, from the pericardium. A tunnel is now burrowed beneath the thymus from top to bottom, and through this is

passed a thread to act as a retractor.   Separation of each lobe margin from its pleural attachment is now undertaken.   The lower edge of a lobe is grasped near to the pleura with fine mouse tooth thumb forceps and separated by gentle traction (see Fig. 2).   The pleural leaf in the rabbit is exceedingly delicate and will rupture at the touch of an instrument.   All dissection must be avoided and separation accomplished by traction alone, although it is of advantage at times to wipe the pleura gently away from the gland with a small wet gauze sponge.   A steady pull upon the lobe margin serves to anchor the gland, and the respiratory undulations and cardiac pulsations furnish sufficient pull to disengage the gland from the pleura.   3 or 4 minutes time may be required for each side.   Should the pleura be injured, the rent must be stopped immediately by a wet sponge to prevent complete collapse of the lung.   Once both lateral margins are free from the pleuræ, there remains only the hilus of each lobe to be dealt with.   By blunt dissection, the fatty tissue in the region of the first rib on either side is cleared away and the glandular vessels brought to view (see Fig. 2).   With these secured the gland may be removed.

Closure of the incision is done in three tiers.   The chest wall is brought together by tying corresponding ligature ends; the divided muscles are united by a running, heavy silk suture; and the skin margins are approximated with a fine silk suture. No dressing need be applied.

Material.

The operation described above was performed on 50 male rabbits; of these 36 were between 1 and 2 years of age, 8 were 3 years old or more, and 6 were between the ages of 4 and 6 months.   36 of the rabbits that survived operation were subsequently inoculated with tumor or *Treponema pallidum;* the others were held for control observations.   The period of observation prior to inoculation was from 10 to 22 days, in most instances 2 to 3 weeks; 29 of the 36 rabbits survived the inoculation and permitted observation over a period of from 2 to 7 months.   The majority of the uninoculated rabbits were observed for approximately 3 months.

RESULTS.

Operative Results.—Among the 50 rabbits on which thymectomy was performed there were 5 deaths related immediately or remotely to the operative procedure.   1 of these was due to air embolism, and the others to shock.   In our last series of 29 operations there was only 1 death; this occurred in an old animal.   Pneumothorax occurred

on the right side in 14 cases, on the left in 4 cases, and bilaterally not at all. There were no ill effects from the pneumothorax.

The chest wall incision united firmly in all of the animals that survived the operation with very little deformity of the thoracic framework and no interference with the function of the fore legs.

Postmortem examination showed that removal of the thymus was complete in all but one animal so far as could be determined, without resorting to microscopic examination. In one rabbit, a small nodule of thymic tissue was found high in the neck; this may have been overlooked at the time of operation. If any fragments were left, they showed no appreciable tendency to hyperplasia. The thymic bed was obliterated by firm fibrous adhesions between the pericardium and the chest wall and the lungs were expanded to their normal proportions.

Postoperative Complications.—Wound infection occurred in 8 animals. Usually an abscess developed within the mediastinum. These infections became encapsulated and pursued a chronic, indolent course which in most cases did not appear to affect the general health of the animals. There were no pulmonary complications, except in the preliminary operations in which intubation of the trachea was done for positive pressure anesthesia. Several of these animals developed pneumonia, atelectasis, or empyema.

Effects of Thymoprivia.—Our study of the effects of thymoprivia was limited to observations on the general physical condition and the reaction of operated rabbits to disease. The results of the latter investigation will be reported elsewhere.

Removal of the thymus produced no appreciable effect on the general health and state of nutrition of the animals. Recovery from the operation was prompt and the operated rabbits showed a gain in weight or a maintenance of weight comparable in all respects to intact normal rabbits. A number of these animals were observed over a period of from 2 to 7 months but during this time no difference in the appearance and physical condition of thymectomized and of normal rabbits was detected. The behavior of young, adult, and old animals which had been operated upon was essentially the same.

DISCUSSION AND CONCLUSIONS.

Thymectomy in the rabbit is a comparatively simple procedure. The open method described above offers many advantages over any of the closed methods and success depends largely upon the observance of a few precautions; chief among these is the avoidance of haste and the observance of aseptic technique.

The operative mortality in our series of rabbits was 10 per cent and there was an additional loss of useful animals, due to secondary complications, amounting to 16 per cent. Among the last 29 rabbits, however, there was only 1 operative death, in an old animal, and 2 cases of slight infection. It appears, therefore, that with some experience the operation can be carried out successfully in nearly all animals, especially so if comparatively young rabbits are used. Old rabbits present greater difficulties on account of the presence of dense fibrous tissue within the field of operation.

While the animals of this series showed no immediate symptoms or alteration in physical condition that could be referred to thymoprivia, it is not to be assumed that removal of the thymus is entirely without effect even on rabbits of mature age. Our observations on the reaction of thymoprivic rabbits to disease show that this is not the case and that in all probability decided effects can be demonstrated if the problem is approached on another basis. The object of the present investigation was, however, to determine whether the thymus could be completely removed from rabbits of certain ages without producing any serious disturbance of health which would interfere with their use in other experiments or complicate the interpretation of results. It was found that this could be done.

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8. Basch, K., *Wien. klin. Woch.*, 1903, xvi, 893.

9. Basch, K., *Jahrb. Kinderheilk.*, 1906, lxiv, 285.

10. MacLennan, A., *Glasgow Med. J.*, 1908, lxx, 98.

11. Ranzi and Tandler, J., *Wien. klin. Woch.*, 1909, xxii, 980.

12. Klose, H., and Vogt, H., *Beitr. klin. Chir.*, 1910, lxix, 1.

13. Klose, H., *Verhandl. deutsch. Ges. Chir.*, 1910, xxxix, 379.

14. Klose, H., Chirurgie der Thymusdrüse, in von Bruns, P., Neue deutsche Chirurgie, Stuttgart, 1912, iii, 283.

15. Klose, H., *Arch. Kinderheilk.*, 1911, lv, 1.

EXPLANATION OF PLATE 1.

FIG. 1. Thymus exposed preparatory to removal.
FIG. 2. Technique employed in removal of the thymus.
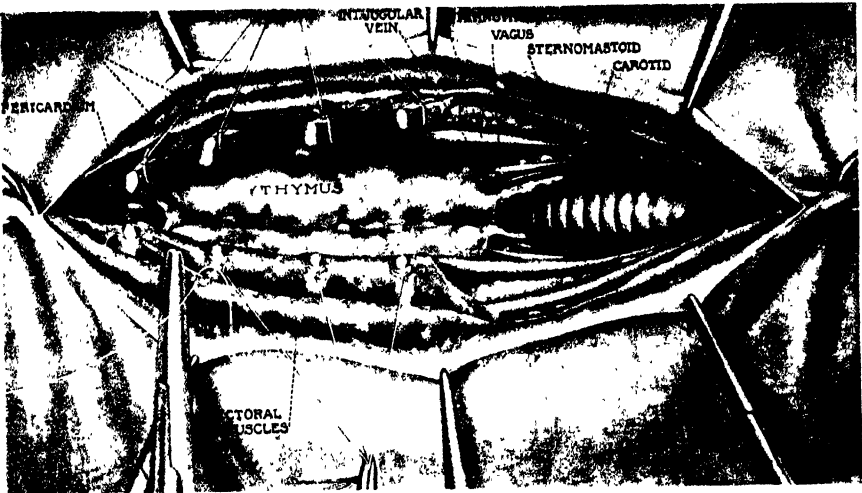
FIG. 1.
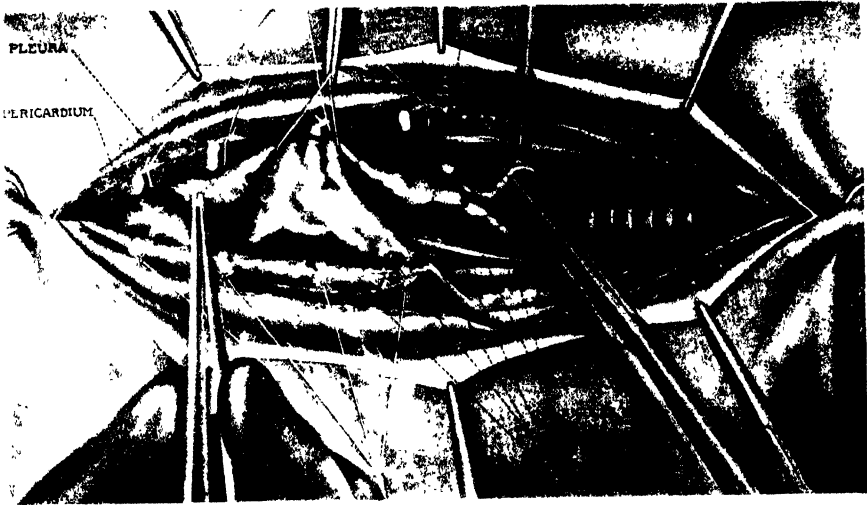
FIG. 2.

ON THE SURFACE COMPOSITION OF NORMAL AND SENSITIZED MAMMALIAN BLOOD CELLS.

By STUART MUDD, M.D., AND EMILY B. H. MUDD.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York, and the Henry Phipps Institute of the University of Pennsylvania, Philadelphia.)

PLATES 2 AND 3.

A simple method has been described for observing the behavior of bacterial or other cells in the boundary surface between two immiscible liquid phases.[1] A theoretical discussion of this behavior has been given, based upon the equilibrium of interfacial tensions involved, and experimental observations concordant with the theory have been adduced.[2] The present communication describes the application of this method to normal and sensitized mammalian blood cells. Striking differences are found in the behavior of erythrocytes after sensitization and of leucocytes after exposure to heat. The results obtained permit certain inferences as to the composition of the cell surfaces and the mechanism of the reactions involved.

Method.

A drop of oil is drawn across a carefully cleaned slide. A small drop of dilute blood cell suspension is drawn along the slide a short distance from and at right angles to the streak of oil. One end of a clean oblong cover-slip is touched to the slide and to the oil so that the oil wets the under surface of the cover-slip along one end (Text-fig. 1). The other end of the cover-slip is now lowered onto the slide, thus spreading the oil into a film under one side of the slip and the blood into a film adjoining it. In the best preparations the blood film does not cover quite all of the area under its end of the slip.

When viewed with the dark-field microscope the interface between the films appears as a bright line with interference fringes, the red cells have their familiar

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[1] Mudd, S., and Mudd, E. B. H., *J. Exp. Med.*, 1924, xl, 633.

[2] Mudd, S., and Mudd, E. B. H., *J. Exp. Med.*, 1924, xl, 647.

dark-ground appearance as shining discs or spheres, and the leucocytes appear as round or irregular cells packed with shimmering granules.   To examine them both the Bausch and Lomb dark-ground condenser with substage 12 candle power bulb and the Zeiss improved paraboloid light- and dark-field condenser with carbon arc have been used satisfactorily.   The Zeiss special apochromatic objective X is satisfactory.

Behavior of Red Cells.

The oil phase ordinarily advances slowly, pushing the aqueous phase ahead of it.   As the interface thus overtakes the suspended or sedimented elements, a characteristic and distinctive behavior of the red and white cells is to be noted.

The erythrocytes are quickly taken into the interface and there, if freely suspended, they are pulled by the liquid-liquid tension into a characteristic lens shape (Fig. 1, *a*).   Unless the interface is advancing
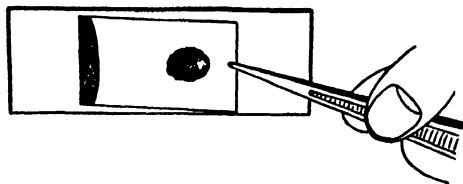
TEXT-FIG. 1.

too rapidly the freely suspended red cells often slide along the interface for a short distance before entering the oil.   Stability of normal erythrocytes in the interface is, however, slight; with the aid of such little mechanical force as is afforded by the slow advance of the interface, they pass readily into the oil phase where they are commonly invisible unless adherent to the glass (Fig. 1, *b*).   Escape from the interface is almost invariably into the oil, rarely into the water. The surface composition of the unsensitized erythrocytes is evidently such that they are more readily miscible with oil than with the aqueous phase but considerably less oil-miscible than are the acid-fast bacteria.[2]

If the liquid-liquid interfacial tension be designated T_{ow}, the cell-oil (solid-organic phase) tension T_{so}, and the tension in the cell-aqueous phase interface, T_{sw}, following the terminology of an earlier paper,[2] it is clear that for unsensitized erythrocytes T_{sw} is greater than T_{so}.   It appears furthermore that neither solid-

liquid tension is greater than the sum of the other solid-liquid plus the liquid-liquid tension, but that the inequality $T_{sw} < T_{so} + T_{ow}$ is small, in some cases almost zero.

These relations explain the observation of Dautwitz and Landsteiner[3] who shook blood cell suspensions with toluene and, on separation of the phases, found the stromata in the toluene phase. The procedure of Dautwitz and Landsteiner might well form the basis of methods of separating a variety of particles with lipoid surfaces from aqueous suspending media.

The erythrocytes studied have been those of the four human groups, of the horse, donkey, sheep, dog, guinea pig, and rat. The test oils used have been Kahlbaum's triolein, tributyrin, and tricaprylin, olive, sperm, cod liver (Norwegian), mineral (Squibbs' Heavy Californian), and cedar oils, and melted vaseline (Chesebrough Manufacturing Co.). Dilute plasma and dilute serum, with and without free hemoglobin, 0.9 per cent NaCl, and Ringer-Locke solution have been used as the aqueous phase. Erythrocytes, freshly drawn or preserved several days, in the form of discs and spheres—more commonly the latter[4]— crenated forms, and stromata have been observed. The relationships described above have appeared regularly under these various circumstances throughout several hundreds of experiments.

Minor variations of course occur and would no doubt be much more in evidence were the method capable of finer quantitative discrimination and were a greater variety of substances used as organic phase. With the present technique, however, variations from the usual behavior appear to be small and for the most part difficult of formulation. The following may be noted.

Among the oils sperm and cod liver oil from another source wet the red cells with perceptibly less ease than the others. A phenomenon we may for brevity call "tails" is particularly likely to occur when the cells are suspended in plasma or when mineral oil is the organic phase. As the red cells in a slowly advancing interface in a thin film rub against the glass the region of contact tends to be drawn out into a process like the neck and stem of a pear provided the cells are sufficiently resistant to wetting by the oil; otherwise they snap over into the oil with very little deformation. These processes, or tails, have been most noticeable with normal cells in films made from plasma of sufficient

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[3] Dautwitz, F., and Landsteiner, K., *Beitr. chem. Physiol. u. Path.*, 1907, ix, 448.

[4] *Cf.* Gough, A., *Biochem. J.*, 1924, xviii, 202; Ponder, E., *Quart. J. Exp. Physiol.*, 1924, xiv, 333.

concentration to clot on standing, but may be seen in less concentrated plasma and serum. The phenomenon is greatly accentuated with sensitized cells, as will appear later.

Red cells in dilute (0.5 to 5.0 per cent) plasma or serum may be pushed for some little distance before a very slowly advancing interface before entering the boundary surface at all. This phenomenon is probably due in part to the interface being occupied by adsorbed protein; it is accentuated in more concentrated sera (5 to 10 per cent). The increased difficulty of passage into and through the interface in concentrated serum and plasma is probably due also in part to condensation of serum proteins upon the red cell surface as shown by Coulter.[5]

Behavior of Sensitized Red Cells.

Red blood cells well sensitized with specific agglutinative and lytic anti-erythrocyte serum differ strikingly from the unsensitized cells in their interfacial reactions. Three phenomena either appear *de novo* or become accentuated with sensitization.

1. The sensitized red cells no longer pass readily from the interface into the oil phase. An interface may advance rapidly and few or no red cells cross from the boundary surface into the oil. Sensitized red cells often slide for long distances along the interface. If such cells do cross into the oil it is with obvious mechanical aid and ordinarily there occurs a perceptible deformation of the interface or of the erythrocyte surface or both.

2. The manifestation already referred to as tails. The sensitized cells agglutinate of course and tend to stick to the glass. As the interface moves by such cells or cell clumps, it tends to carry the cells with it since they resist wetting by the oil phase. The cells are often anchored by their points of contact with the glass. The result is that pear-shaped (Fig. 2) or filamentous (Figs. 5 and 6) prolongations of the red cell surfaces are produced. If the red cell is anchored by a larger area a dumb-bell shape may result; such a cell has even been observed to pinch in two, each half cell then rounding up.

This tail phenomenon is beautifully, though faintly, shown in the photographs of Fig. 5, but has been almost lost in reproduction. Comet-like streamers are just discernible stretching from the cell clump in the center of the field toward the lower right hand corner. From the single cell between the retreating interface (upper left hand corner)

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[5] Coulter, C. B., *J. Gen. Physiol.*, 1921–22, iv, 403.

and the cell clump in the center, two tails forming an inverted U may just be seen pointed downward and slightly to the right (Fig. 5, *c*).

3. The interface is often bent backward in passing over very small cell clumps or even single sensitized cells.

Figs. 3, 5, and 6 are enlargements from moving picture films of such phenomena. The direction of advance of the interface is indicated in each case by the arrow. In Fig. 3 the advancing interface has been blocked locally by a clump of sensitized red cells adherent to the glass and has been bent backward into a right angle. In Fig. 5 a similar occurrence is carried a step further. The oil phase advancing on the aqueous phase (upper left hand corner) with its suspended blood cells meets groups of sensitized red cells it cannot easily wet. The interface, stopped locally, advances elsewhere and thus leaves a clump of agglutinated cells in aqueous salt solution isolated within the oil (Fig. 5, *a* and *b*); streamers behind the clumped cells indicate how far they have been dragged by the advancing interface from their original site of attachment to the glass. Still retarded by a small group of cells the interface is dragged over one or more of them (Fig. 5, *b* and *c*) and then retracts according to the law of minimal areas.[6] One erythrocyte with tails remains stuck to the glass and visible, the others are either suspended in the oil and hence invisible or have been carried out of the field on the aqueous side of the retracting interface. Fig. 6 shows a clump of agglutinated red cells as it is left behind by the retreating interface; tails are here more plainly shown.

Specific sensitization of the erythrocytes, then, stabilizes them in the interface between organic and aqueous phases; this effect is the result of an alteration of the red cell surfaces by the sensitizing agent such as to make them more difficultly miscible with oil. The predominantly non-polar[7] surface of the unsensitized erythrocyte thus becomes with sensitization more polar. In terms of interfacial tension, specific sensitization increases the inequality $T_{sw} < T_{so} + T_{ow}$ and decreases the inequality $T_{so} < T_{sw} + T_{ow}$. This observation is in harmony with that of Meyer[8] who found hemolytic amboceptors to be insoluble in lipoid solvents. The further significance of the alteration of the erythrocyte surface with sensitization will be discussed below.

This altered interfacial behavior, or "stabilization reaction" as

[6] Plateau, J., Statique des liquides soumis aux seules forces moléculaires, Paris, 1873, 9.

[7] Lewis, G. N., *J. Am. Chem. Soc.*, 1913, xxxv, 1448; 1916, xxxviii, 762. Harkins, W. D., in Holmes, H. N., Colloid symposium monograph, New York, 1925, ii, 163.

[8] Meyer, K., *Arch. Hyg.*, 1908, lxvii, 114.

it may be called, is less sensitive than lysis or agglutination. The red cells to be tested are washed several times with Ringer-Locke or physiological NaCl solution, mixed with the specific anti-erythrocyte serum in proper dilution, and allowed to stand in the ice box usually for an hour or more. Tested then the altered interfacial behavior has been found with the following concentrations of immune serum, the homologous red cells being each time in 1 per cent suspension: anti human-erythrocyte rabbit serum (obtained through the kindness of Dr. Hideyo Noguchi) 0.3 per cent and higher; anti horse-erythrocyte rabbit sera,[9] Nos. 32 and 40, 2.0 per cent and higher, Nos. 15 and 30, a few tenths per cent and higher.[10] Unwashed horse cells in anti-horse sera 16 and 31 gave some positive and some negative stabilization reactions in concentrations of 2 to 6 per cent. Titers with unwashed cells were not constant, however.

Cross-tests with these same immune sera do not yield the stabilization reaction. Thus the anti-human serum in 5 per cent concentration gave negative tests with 1 per cent horse cells. 1 per cent suspensions of human cells tested with 20 per cent anti-horse serum 40 and with 4.8 per cent anti-horse serum 15 were not stabilized. At approximately the same concentrations and at lower ones these sera gave strong positive reactions with homologous cells. Rat cells, 1 per cent, . in 3 to 7 per cent anti pony-erythrocyte rabbit serum were not stabilized in the majority of experiments; but in three tests the results were weakly positive.

Through the kindness of Dr. K. Landsteiner and Mr. J. van der Scheer, it has been possible to carry a step further the analysis of the action of immune serum upon the erythrocyte surface. These investigators, using alcohol extracts of red cells as antigens, have obtained

[9] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1925, xli, 427. The numbers we have given the anti horse-erythrocyte sera are taken from this paper.

[10] The high titers found for Sera 15 and 30 as compared with those previously found with Nos. 32 and 40 were unexpected. The explanation may well have been in the difference in the solutions in which the test red cell suspensions were made; *i.e.*, physiological NaCl in the later and Ringer-Locke solution in the earlier experiments. The reaction of the Locke solution as we made it up was about pH = 8.7. Coulter (Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 513) has shown that considerably less sensitizer is combined with the red cells at alkaline reactions than at the optimal weakly acid reaction.

anti-erythrocyte sera with a ratio of the lysin to agglutinin titers of about 1:1–1.5 instead of 1:4–8 as with sera obtained after injection of whole erythrocytes.[9] Two pair of sera, with one extract immune serum and one whole erythrocyte immune serum in each pair, have

TABLE I.

Organic phase.	Cell concentration.	Serum concentration.	Cells stabilized by serum.		Remarks.
			No. 40. Extract immune serum.	No. 32. Whole red blood corpuscle immune serum.	
	per cent	*per cent*			
Cod liver oil.........	0.5	10.0		+	
" " "	1.0	5.0	+	+	
" " "	1.0	4.0	(±)		Test within a few min. of making preparation. See Rows 11 and 12 below.
" " "	1.0	4.0		+	
" " "	1.0	3.6	+	+	
Mineral "	1.0	3.6	−		
Cod liver "	1.0	3.6	+		
Olive oil.............	1.0	2.8	+	+	
Cod liver oil.........	1.0	2.8	±	±	
" " "	1.0	2.5		±	
" " "	1.0	2.0	±		Test within a few min. of making preparation.
" " "	1.0	2.0	+	+	Same preparation 90 min. later.
" " "	1.0	2.0	±	±	
" " "	1.0	1.2	−	±	
Mineral "	1.0	1.2	−	−	
Cod liver "	1.0	1.2	−	−	
Olive oil.............	1.0	1.2		−	
" "	1.0	0.6		−	

been tested for stabilization reaction titers. The two sera of each pair were tested at any given concentration with the same red cell suspension and with experimental conditions as nearly identical as possible. The tests with one pair are given in Table I.

The stabilization titers are evidently about the same for the two

sera as are also the lysin titers determined by Landsteiner and van der Scheer.[9] The agglutination titers on the other hand are quite different. The results with this and the other pair of sera are summarized in Table II.

It will be appreciated that the criteria for the stabilization reaction cannot be applied with quantitative precision, and that the ratios given are approximate only. Nevertheless we are convinced of the correspondence of the stabilization and lysin ratios within the rather large error of the method and of the failure of the stabilization and agglutination ratios to correspond even approximately within the error of the method. Corroborative evidence is furnished by the fact

TABLE II.

Agglutinin titer,	Serum 40:	Serum	32 = 1:6–8
Lysin titer,	" 40:	" 32 = 1:1
Stabilization titer,	" 40:	" 32 = 1:1
Agglutinin "	" 15:	" 30 = 1:8
Lysin titer,	" 15:	" 30 = 1:2
Stabilization titer,	" 15:	" 30 = 1:1–3

Nos. 40 and 15 extract immune sera; Nos. 32 and 30 whole erythrocyte immune sera.

Sensitivity, stabilization reaction : lysis = 1:3–8 (*i.e.* lysis was obtained by Landsteiner and van der Scheer with serum dilutions 3 to 8 times those required to give the stabilization reaction).

that there was agglutination of the red cells suspended in the whole erythrocyte antisera even when the stabilization reaction was negative. Agglutination was regularly much less in the corresponding extract immune sera suspensions, and sometimes gross agglutination or even occasionally microscopic agglutination could not be detected when the stabilization reaction was positive.

The altered behavior of sensitized horse erythrocytes thus depends predominantly upon the hemolytic sensitizer rather than upon the the agglutinin. It is difficult to escape the conclusion (already reached by Landsteiner and his coworkers on other grounds[9]) that the hemolytic sensitizer reacts predominantly with lipoid components of the red cell surface. It would seem also that the agglutinin reacts predominantly with the protein moiety of the erythrocyte surface.

Specifically hemolyzed stromata were prepared by making a 2 per cent suspension of human cells in 4 per cent anti human-erythrocyte rabbit serum and adding fresh human whole blood. The resulting ghosts behaved like strongly sensitized unhemolyzed cells. Even single ghosts bent back the interface. Stromata resulting from 1 per cent horse cell suspensions in 6 per cent anti-horse sera 16 and 31 mixed with equal volumes of 10 per cent fresh guinea pig serum likewise showed strong stabilization reactions.[11] In contrast to the altered interfacial behavior of the strongly sensitized stromata was the normal behavior of stromata obtained by hemolysis with distilled water or by cold during an overnight stay in the ice box. The unsensitized ghosts went over into the oils as easily as ordinary red cells or more so.

Behavior of Leucocytes and Platelets.

During the observation of preparations with the Bausch and Lomb dark-field condenser with substage lamp the appearance and behavior of the human leucocytes encountered was as follows:

1. Cells rounded in outline in which granules could often be observed in movement. These cells entered the boundary surface with little difficulty and were spread along the interface with complete disintegration. Some of the component granules streamed along the interface in both directions away from the site of disintegration, where the liquid-liquid interfacial tension was obviously lowered, and others passed over into the oil. Less frequently a rapidly advancing interface swept over such a leucocyte, leaving it somewhat spread and disintegrated in the oil.

2. Cells irregular in outline in which movement of granules was not seen. Such cells were often pushed for many minutes before the advancing interface. If the white cell was stopped by adhesion to the glass or was overtaken by a too rapidly advancing interface it bent the interface back into a "peninsula" before being dragged into the oil. Often the leucocyte in the distal end of such a peninsula was

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[11] Apparently negative reactions were obtained, however, with some ghosts from partially hemolyzed horse cell-antisera mixtures in which the unhemolyzed cells appeared to give positive reactions. '

left in the oil surrounded by a film of aqueous phase.   A peninsula about to break off thus is shown in Fig. 4; the leucocyte has been partially dragged out of the vacuole into the oil.

Leucocytes in preparations examined over the Zeiss condenser with carbon arc and heat filter showed almost exclusively the behavior described under (1) above.   An illustrative protocol is given.

Small drop of Ringer-Tyrode solution put on slide; tiny drop of blood fresh from finger added. Oil drop put on other half of slide and cover-slip superposed. Preparation observed with Zeiss condenser (bright field, which shows better than the dark field the distinctions between the leucocyte cell types), arc, and heat filter. Red cells seen to snap across into oil in usual way. Many white cells studied, mostly granular leucocytes; these were in healthy condition. When hit by interface they entered it, formed lenses, and disintegrated, spreading up and down interface; when this happened the interface would run forward locally as disintegrated protoplasm spread. Some white cells formed lenses and passed directly into oil, disintegrating as they were enveloped by the oil. An eosinophil was seen to enter the interface, form a lens, and slide along the interface about one oil immersion field; it then spread in the interface and some of the large eosinophil granules drifted over into the oil. The preparation was now transferred to the microscope with substage lamp. At first a few normal looking polymorphonuclear leucocytes were seen to act in the manner described in the first part of the experiment but gradually they came to look more pronouncedly granular and less liquid, more hard and irregular in outline. As this process progressed the cells at first bent back the interface very slightly and were disintegrated as they passed into the oil; then bent the interface back more and dragged through into the oil, and finally acted in the typical manner described under (2) above.

The altered behavior of leucocytes viewed with the substage lamp appears to be due to the heating of the film by this type of illumination.   A thermal junction (made by soldering together constantan No. 40 and copper No. 32 wire and flattening with a hammer) was introduced into films like those described; the other junction was put in ice water, and the electromotive force determined with a potentiometer.   The temperatures reached in three different experiments over the condenser with substage lamp were: 53.8°C. in 29 minutes, 57.8° in 44 minutes, 58.9° in 33 minutes.   The first preparation only reached 2^c or 3° above room temperature when illumined by the carbon arc with water trough heat filter.

Behavior (1) may be regarded as normal for white blood cells under

the experimental conditions described and behavior (2) as that of heat-fixed white cells. The surface tension relations could not with certainty be determined for the normal leucocytes because of their inability to resist spreading. Our belief is, however, that the conditions of equilibrium in the interface are fulfilled; i.e., $T_{so} < T_{sw} + T_{ow}$ and $T_{sw} < T_{so} + T_{ow}$. After heat fixation the leucocytes are extraordinarily resistant to any wetting whatsoever by the oil; they are apparently not in equilibrium in the interface, and $T_{so} > T_{sw} + T_{ow}$.[12] Human leucocytes thus appear to be more labile than erythrocytes both in their resistance to the interfacial stresses and in their resistance to surface alteration with elevation of temperature. We have not adequately studied leucocytes of other species. Kite[13] from micro dissection studies has also concluded that leucocytes are naked protoplasm unprotected by any morphologically differentiated surface layer.

Rabbit blood platelets, like the human leucocytes, became increasingly refractory to wetting by the oils (mineral and cod liver oils and melted vaseline) during exposure to the heat of the substage lamp.

Fresh platelets, prepared by drawing 4 cc. of rabbit blood directly into 1 cc. of 1.3 per cent sodium oxalate solution, and mixing and separating the platelets by centrifugation, were made available daily through the kindness of Dr. C. M. Van Allen. Diluted with NaCl and studied with the heat filter or during the first minutes of observation with substage lamp, the platelets floated through the interface as though it offered little if any obstruction. The platelets did not form lenses or spread in the interface. After a few minutes over the substage lamp the platelets were much more stable in the interface. They slid along the interface for longer distances, left it only at the expense of mechanical work, and bent back the interface in so doing.

Minute, brightly shining granules were seen regularly in the preparations of blood and serum. These adhered readily to the glass and

[12] This result is remarkable in that the alteration of the leucocytes with heating is in just the opposite sense to that which would be anticipated from denaturation of the proteins of the protoplasm (Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1925–26, ix, 169). It suggests either a redistribution of substances in the surface layers of the cell or chemical modification of non-polar surface groups to form polar groups.

[13] Kite, G. L., *J. Infect. Dis.*, 1914, xv, 319.

when overtaken by the interface pulled it into sharp pointed peninsulas. One of these is shown in the middle of the interfacial line in Fig. 1, *a* and *b*, and two appear in the upper left hand corner of Fig. 4. The behavior of these granules in the interfaces shows their surfaces at least not to be fat-like. They seem to be among those comprised under the term hemokoniæ or blood dust.[14] However, they are clearly different from the spherules seen in the turbid serum of animals bled without having been previously fasted (alimentary lipemia). The existence of these two types of refractile particles in sera doubtless accounts for some of the confusion in the literature regarding hemokoniæ.

Behavior of Some Other Particles.

Pure crystals of oxyhemoglobin given us by Dr. M. Heidelberger were suspended in distilled water and studied against the oils (triolein, tricaprylin, mineral, cod liver, sperm, and cedar oils). They were stable in the interfaces, slid along them, and bent back the boundary line when dragged through into the oils.

Potato-starch (Kahlbaum's) and corn-starch grains suspended in distilled water were swept before the advancing oils (triolein, tricaprylin, mineral, and cod liver oils). They were not stable in the interfaces and were in no instance observed to cross into the oil except when a vacuole of water containing starch grains was left behind at the end of a peninsula.

Shreds of ground up filter paper (Baker and Adamson, ash-free) in distilled water were pushed before the interfaces and bent the interface into peninsulas if dragged through into the oils (triolein, tricaprylin, tributyrin, mineral, and cod liver oils).

The interfacial tension relations of a number of cells and cell products in aqueous suspension in contact with the specified organic phases (references 1 and 2 and this paper) fall into the following categories.

[14] Krehl, L., and Marchand F., Handbuch der allgemeinen Pathologie, Leipsic, 1912, ii, pt. 1, 215. Hewlett A. W., Functional pathology of internal diseases, New York and London, 1917, 243. Stengel, A., and Fox, H., A textbook of pathology, Philadelphia and London, 7th edition, 1921, 436.

1. These particles are wet by water but not by oil. They are not in equilibrium in the organic-aqueous phase interface. $T_{so} > T_{sw} + T_{ow}$:

Heat-fixed leucocytes; starch grains; certain granules in serum; cellulose (filter paper) (?).

2. These particles are in equilibrium in the organic-aqueous phase interface. $T_{so} < T_{sw} + T_{ow}$ and $T_{sw} < T_{so} + T_{ow}$:

(a) Escape into the aqueous phase is relatively easy. The inequality $T_{so} < T_{sw} + T_{ow}$ is small. $T_{so} > T_{sw}$:

Ordinary bacteria;[2] mammalian erythrocytes and stromata after strong specific sensitization; oxyhemoglobin crystals (?); cellulose (?).

(b) Relative ease of escape into organic and aqueous phase not certain:

Fresh human leucocytes; heated rabbit platelets; oxyhemoglobin crystals (?).

(c) Escape into oil is relatively easy. The inequality $T_{sw} < T_{so} + T_{ow}$ is small. $T_{sw} > T_{so}$:

Acid-fast bacteria against most oils; unsensitized mammalian erythrocytes and stromata; some preparations of fresh oxalated rabbit platelets.

3. These particles are wet by the organic phase but not by the aqueous phase. They are not in equilibrium in the interface. $T_{sw} > T_{so} + T_{ow}$:

Acid-fast bacteria with oleic acid and tricaprylin; some preparations of un-heated oxalated rabbit platelets (?).

DISCUSSION.

Mammalian erythrocytes possess a surface pellicle[15] which often becomes wrinkled in our films. Such cells may be seen to be pulled out by the interfacial stresses into lens-shaped bodies with smooth surfaces. The red cells are not spread with disintegration by the interfacial stresses as are normal human leucocytes. As to whether all or only a part of the erythrocyte pellicle is concerned with the cell permeability the present work affords no data.

The surface of erythrocytes is capable of being drawn out into delicate processes. These have been described previously by Noguchi[16] with cells in process of hemolysis by snake venom, by Kite[13] and by Mathews[17] with untreated cells, and by Rous and Robertson[18] with human red cells more especially from patients with pernicious anemia.[19]

[15] Ponder, E., *Proc. Roy. Soc. London, Series B*, 1924–25, xcvii, 138.

[16] Noguchi, H., Snake venoms, *Carnegie Institution of Washington, Pub. No. 111*, 1909, 170.

[17] Mathews, A. P., Physiological chemistry, New York, 2nd edition, 1916, 465.

[18] Rous, P., and Robertson, O. H., *J. Exp. Med.*, 1917, xxv, 651.

[19] Rous, P., personal communication; also *Physiol. Rev.*, 1923, iii, 75.

Such processes may also be seen extending from the distorted stromata obtained by prolonged centrifugation at high speed of laked blood. The formation of surface processes in our preparations is greatly accentuated by treatment with specific anti-erythrocyte serum.

In an interesting study on the electric capacity of blood, Fricke[20] has lately drawn the conclusion, with the aid of certain additional assumptions, that the surface membrane of mammalian erythrocytes is only monomolecular in thickness. This view we cannot harmonize with the facts just mentioned. Moreover, Seifriz[21] has been able to stretch the stromata between micro dissection needles; from an amphibian erythrocyte he can actually release cytoplasm and nucleus and then stretch the elastic pellicle.

Analytical studies have shown that the stromata of erythrocytes contain mineral salts, protein, and lipoid, cholesterol and lecithin constituting the greater part of the lipoid.[22] The presence in the pellicle of lipoid and protein is also indicated by ultramicroscopic observation.[23] This makes it probable although not certain that normal red cells present to the plasma surfaces with both lipoid and protein components.

Evidence indicating the presence of protein in the erythrocyte surface is furnished by cataphoretic and titration data of Coulter's[24] showing an isoelectric point at pH = 4.6. The shape of the titration curves and the position of the isoelectric point[25] suggest that material other than the stroma globulin is also present in the surface. The well known fact that substances which attack lipoids are hemolytic also bespeaks a part for lipoids in the erythrocyte surface.

The work of Bang and Forssman, Landsteiner and his collaborators, and others[9] indicates also the dual nature of the antigens of the red cell surfaces. Alcohol and ether extracts of red cells give rise to and react with antisera with relatively small agglutinin:lysin ratios; the lipoid-insoluble residues show protein reactions and give rise to agglutinins.

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[20] Fricke, H., *J. Gen. Physiol.*, 1925–26, ix, 137.

[21] Seifriz, W., personal communication.

[22] Pascucci, O., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 543, 552. Wells, H. G., Chemical pathology, Philadelphia and London, 4th edition, 1920, 211. Hamburger, H. J., *Ergebn. Physiol.*, 1924, xxiii, pt. 1, 129.

[23] Salén, E., *Biochem. Z.*, 1920, cx, 176.

[24] Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 309.

[25] Mudd, S., *J. Gen. Physiol.*, 1924–25, vii, 389.

Further corroborative evidence is furnished by our observation that normal erythrocytes pass readily but not spontaneously from the interfaces into oil; that specifically sensitized erythrocytes are no longer readily miscible with oil; and that this increase in polarity of the erythrocyte surface on sensitization corresponds fairly well to the hemolysin titer ratios and is relatively independent of agglutinin titer.

The recent contention of Gorter and Grendel[26] that erythrocytes are covered with a layer of lipoids just two molecules thick, aside from its *a priori* improbability, is in direct conflict with several of the above facts. It is clear that the unsensitized erythrocyte surface contains both lipoid and protein. The changes produced in this surface by treatment with immune sera have already been discussed.

SUMMARY.

The interfacial tension method has been applied to the study of the surface composition of mammalian blood cells and to certain other particles.

Unsensitized erythrocytes and stromata possess only a small margin of stability in the interface and pass readily into the oil phase.

Specifically sensitized erythrocytes and stromata possess much greater stability in the interface and pass into the oil only with considerable mechanical aid; characteristic deformations of the erythrocyte surface or the interface or both often result.

With special immune sera prepared by Landsteiner and van der Scheer[9] the quantitative relations are such as to indicate that the increased polarity of the sensitized erythrocyte surface is due to combination of the red cell surface lipoids with hemolytic sensitizer. These results are corroborative of the conclusion of Landsteiner and van der Scheer that erythrocytes contain specific lipoid-soluble antigens.

The tentative conclusion is reached that with these anti horse-erythrocyte sera at least the agglutinins combine predominantly with the protein of the red cell surfaces.

Fresh human leucocytes are spread and disintegrated by the interfacial stresses. After heat injury over the condenser with substage

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[26] Gorter, E., and Grendel, F., *J. Exp. Med.*, 1925, xli, 439.

lamp the leucocytes typically do not enter the boundary surface. They are pushed before the advancing interface and, if their further advance is obstructed, bend the interface backward to form peninsulas and vacuoles. This change after heating is in the opposite sense to that to be expected from denaturation of the proteins of the protoplasm.

Fresh oxalated rabbit platelets pass very easily into the oils. After heating over the substage lamp these elements also become less oil-miscible.

The interfacial tension relations of blood cells, bacteria, and several cell products are tabulated.

We would express our gratitude to the investigators who have given us difficultly obtainable materials and valuable advice; and our appreciation also of the skill of Mr. H. Rosenberger and Mr. L. Schmidt in taking the dark-field moving pictures.

EXPLANATION OF PLATES.

PLATE 2.

FIG. 1. Cinematograph of normal red blood cell passing through interface. Cod liver oil on left; human blood diluted with 0.9 per cent NaCl on right; bright line, boundary surface between them. Arrow indicates direction in which interface is advancing.

FIG. 2. Sensitized red cells. Cod liver oil on right; horse cells, 0.5 per cent, in 33 per cent anti horse-erythrocyte rabbit serum, on left. Two pear-shaped red cells in interface. Whole field slightly out of focus.

FIG. 3. Clump of sensitized red cells bending back interface. Slightly out of focus.

FIG. 4. Heated human leucocyte in a vacuole of dilute plasma about to be left behind in olive oil.

PLATE 3.

FIG. 5. Cinematograph of interface leaving a vacuole behind and dragging over a small clump of sensitized red cells. Triolein against 1 per cent suspension of horse cells in 0.8 per cent anti horse-erythrocyte serum 30.

FIG. 6. Vacuole and tails. Same materials as in Fig. 5.

The oblong spaces at the sides of Figs. 2, 3, and 4 are perforations in the moving picture film.
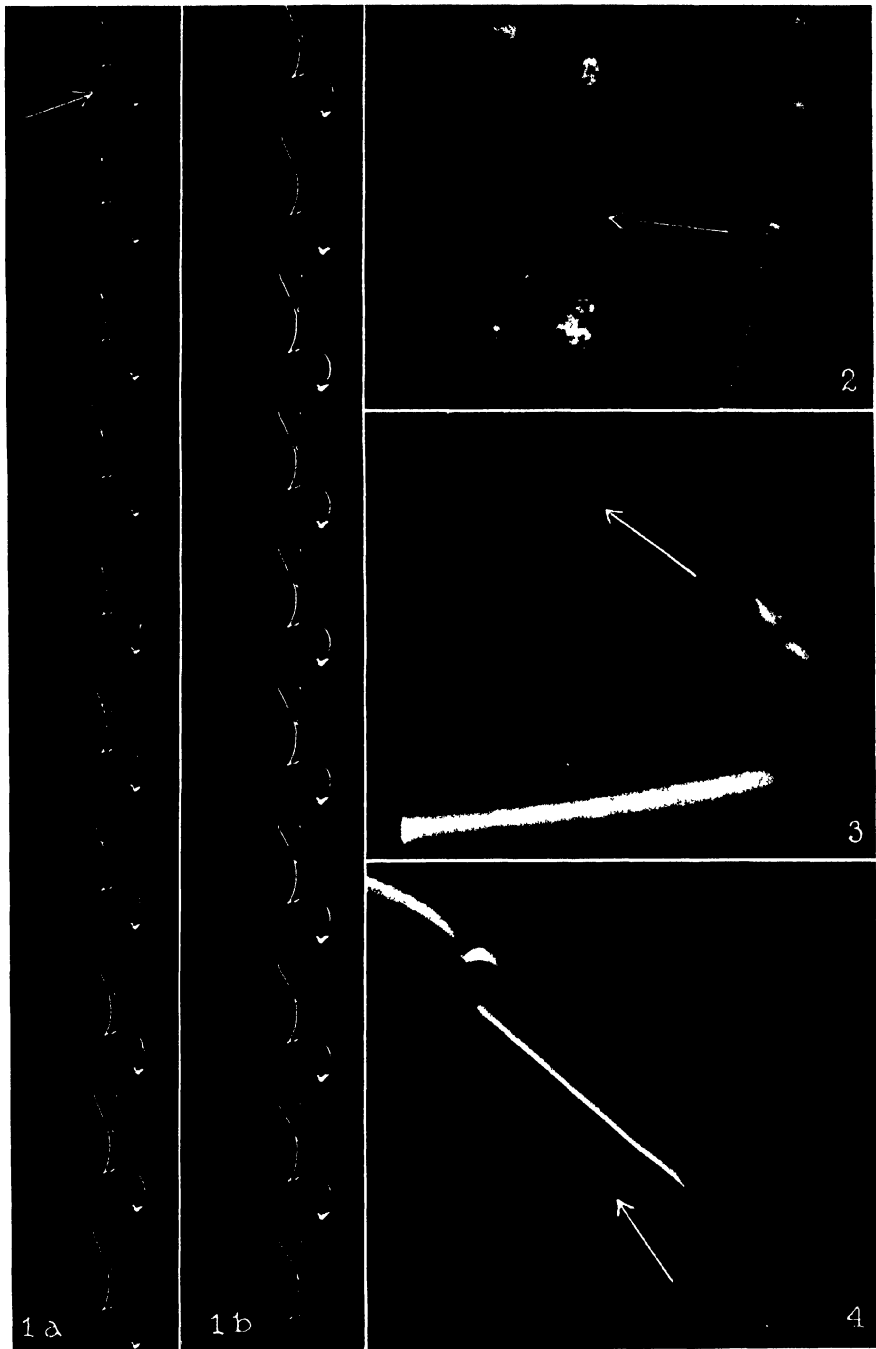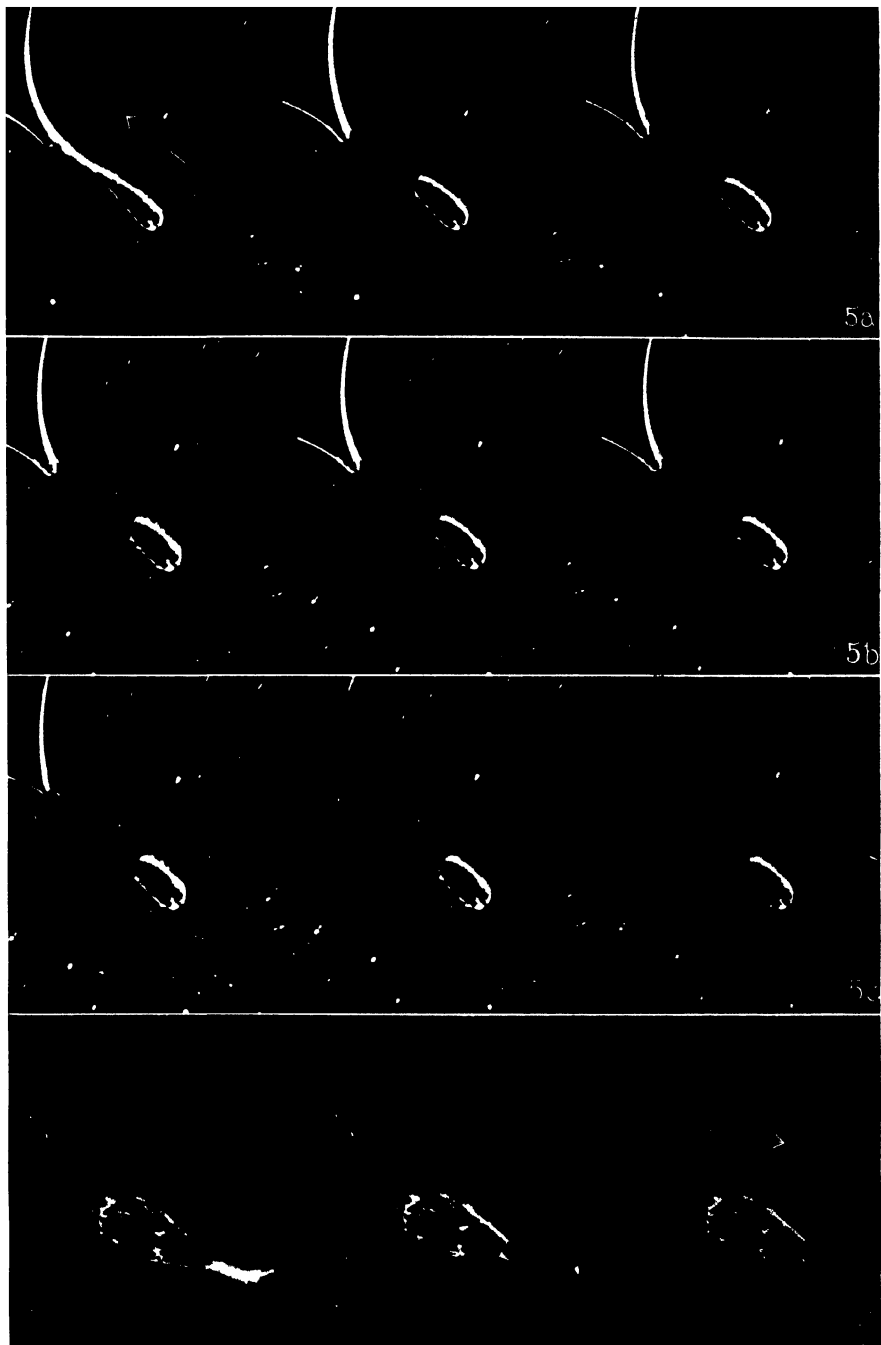
ELECTROENDOSMOSIS THROUGH MAMMALIAN SEROUS MEMBRANES.

III. The Relation of Current Strength and Specific Resistance to Rate of Liquid Transport. Transport Rate with Serum.

By STUART MUDD.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The present experiments concern the relation of the electric current strength and of the specific electrical resistance of the perfusing liquid to the rate of electroendosmotic flow across mammalian serous membranes. A direct proportionality is found between liquid and electric flow through these membranes, which are complex in structure and heterogeneous in composition. The variables Φ and I are thus connected in the complex case by the same relationship as in the case of simple membranes to which the classical electroendosmotic equation applies. Less simple relations are found when the membranes are bathed in buffers of varying specific resistance. Quantitative determinations are also reported with whole serum and the membranes of living and dead animals. The rate of electroendosmotic flow across dog and cat serosæ bathed in serum has been found to be 0.2 to 0.3 c.mm. per minute per milliampere toward the cathode.[1]

Relation of Current Strength to Rate of Flow.

The experimental set-up is shown in Fig. 1. The membranes were fastened by broad rubber bands over the mouth of the electrode vessel; the inside diameter

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[1] The ratio of current strength to liquid flow through any given membrane is independent of the area of membrane through which the current is passing. For if a given constant potential difference is maintained across the membrane, the current strength and the volume of liquid transported in unit time will both be proportional to the area of the membrane through which flow occurs. The dimension of area therefore cancels out and does not appear in the ratio of liquid to electric flow.
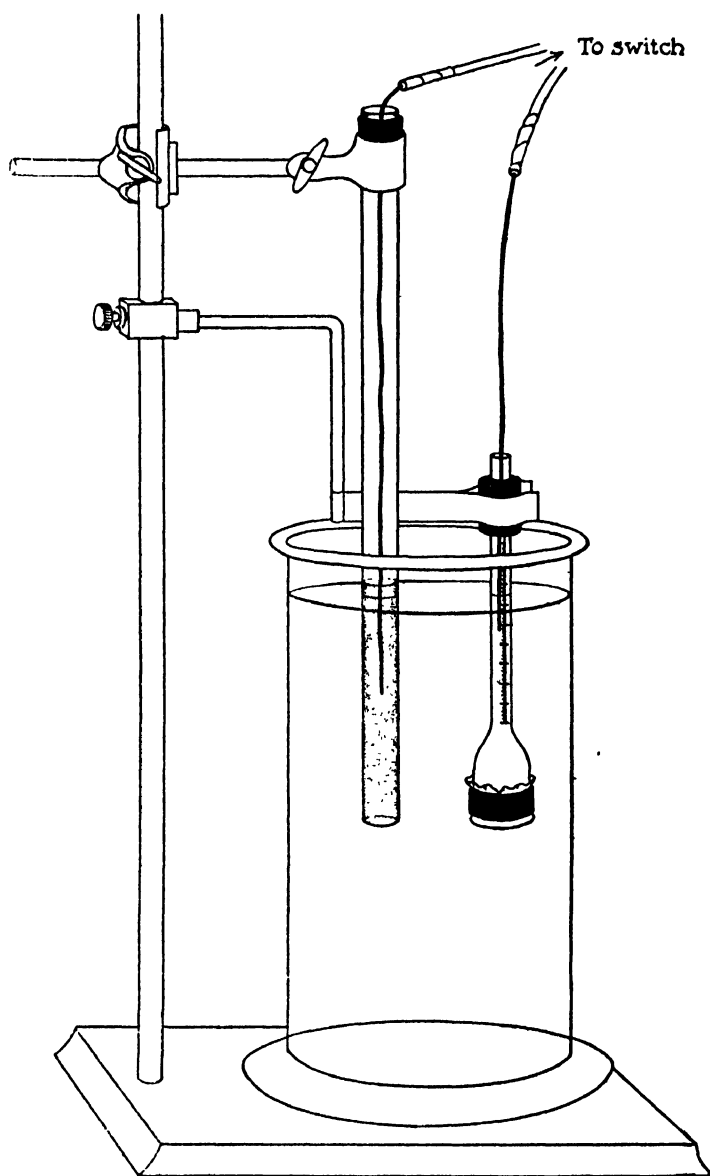
FIG. 1. Arrangement for study of electroendosmotic transport across animal pericardia.   See text.

of the mouth was 18 mm.   The electrode vessel was filled and emptied with capillary pipettes; it dipped into a large vessel of buffer containing an agar electrode. Current was led into the buffer in the electrode vessel by a platinum wire.   The buffer within and without the electrode vessel was the same.

The source of E.M.F. was connected to the two ends of a 666 ohm slide-wire rheostat.   The experimental lines were led off, one from one end of the rheostat and the other from the rheostat slider.   By moving the slider, therefore, the
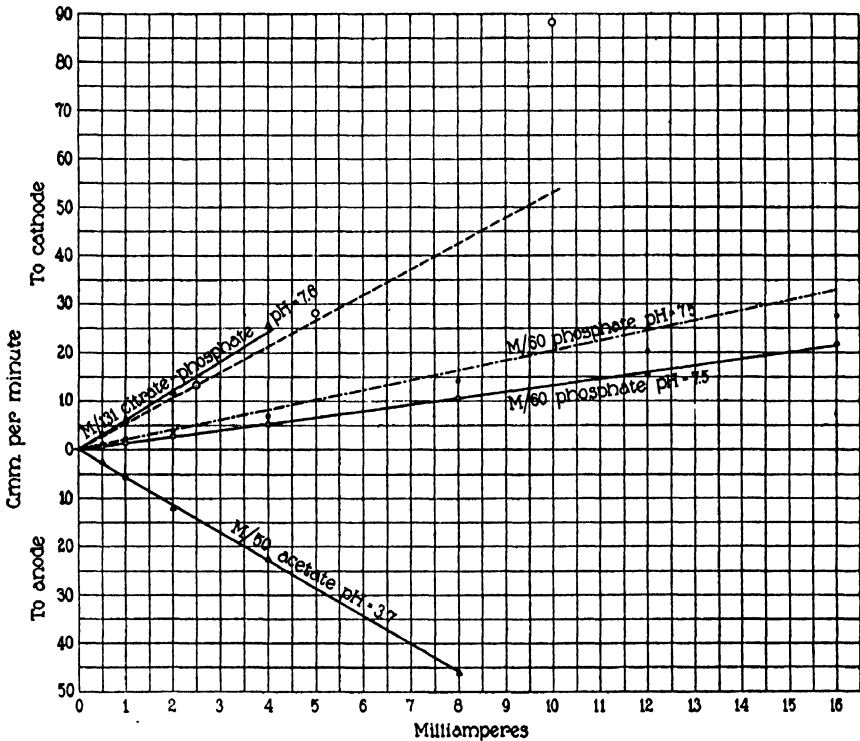
FIG. 2. The relation between volume of liquid transported in unit time (ordinates) and electric current strength (abscissæ) for several systems.

P.D. between the experimental lines could be varied continuously from zero to the total applied E.M.F.   A pole-charging switch and a Weston standard milliammeter were included in the circuit.   The experimental procedure finally adopted gave satisfactorily reproducible results with a minimum of error due to temperature changes, leakage, bulging of the membrane, polarization, etc.

The data obtained are presented in Fig. 2.   The points fall with considerable accuracy along the solid straight lines, and these pass

through the origin; the proportionality of liquid and electric flow even with minimal current strengths is thus indicated.

The points do not fall satisfactorily along the broken lines.   In the case of the dash line (M/131 citrate-phosphate data) the erratic position of the points above 4.0 milliamperes is almost certainly due to the effect upon the membranes of chemical changes about the platinum electrode.   In these experiments the electrode was brought near the membrane in order to obtain the desired current strengths.   Later the electrode was kept well up in the narrow vertical tube.

In the case of the broken line with M/60 phosphate mixture, a disturbing factor seems to have been that the membranes were applied very laxly over the mouth of the electrode vessel.

The slopes of the straight lines indicate the rate of liquid transport per milliampere characteristic for each system.   These are:

Buffer mixture of Na$_2$HPO$_4$ and citric acid; molarity, M/131; pH 7.6.   Solid line, composite of two experiments with lean cat pericardia.   Rate of liquid transport, 6.00 c.mm. per milliampere per minute to cathode.   Dash line, composite of three experiments with cat pericardia.

Buffer mixture of Na$_2$HPO$_4$ and KH$_2$PO$_4$; molarity, M/60; pH 7.5.   Solid line, lean pericardium of male dog; runs continued throughout 6 working days. Rate of liquid transport, 1.33 c.mm. per milliampere per minute to cathode. Experimental site at end of experiment composed chiefly of bundles of collagen fibres in two more or less well defined lamellæ; elastin fibres among collagen bundles; basement membrane persistent only in places; mesothelium gone; in one region a zone of looser connective tissue containing fat cells, blood vessels, and a nerve.   Fibre bundles considerably frayed out.   Dot-dash line, composite of three experiments with dog pericardia; a few runs only with each membrane.

Buffer mixture of acetic acid and sodium acetate; molarity, M/50; pH 3.7. Lean pericardium of male dog; runs through 4½ working days.   Rate of liquid transport, 5.76 c.mm. per milliampere per minute to anode.   Experimental site of dense zones of collagen fibres with a few elastin fibres interspersed; in a part of section looser connective tissues containing fat cells and blood vessels.   Basement membrane persistent in places only; mesothelium gone.

Relation of Specific Resistance of Buffer to Rate of Flow.

The buffers used were Sörensen's phosphate mixtures to which NaCl was added to give the desired conductivity.   The stock KH$_2$PO$_4$ and Na$_2$HPO$_4$ solutions were mixed and diluted to give buffers of approximately 7.4 pH and M/60 concentration.   Sodium chloride was added in amounts such as to give a series of M/60, M/50, M/40,

M/30, M/20, M/10, M/7, and M/6 total molar concentration. The pH was readjusted to about 7.4 by addition of dilute NaOH. The specific resistance of the buffers was determined at 21.6° ± 0.2°C. by the ordinary Kohlrausch method. The viscosity of the M/6 buffer was found to be only a little more than 2 per cent greater than that of distilled water. Viscosity measurements were thereafter
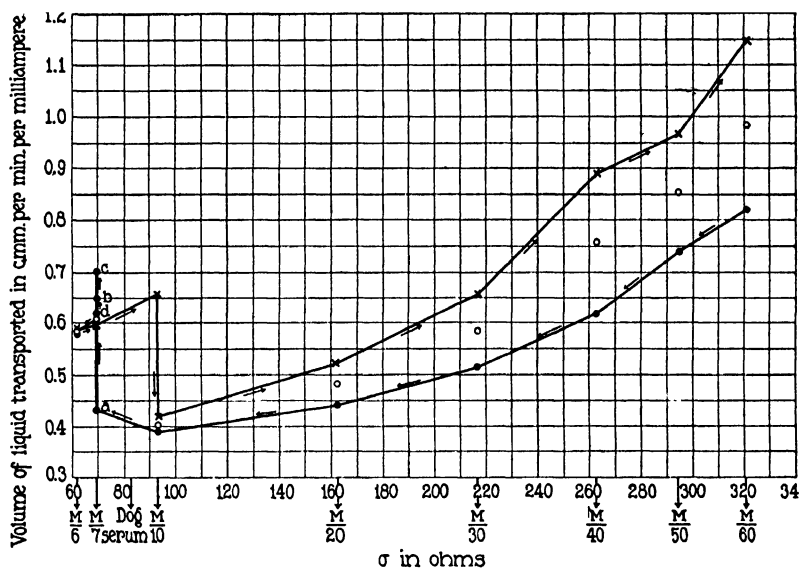
FIG. 3. Experiment 1. Relation of rate of electroendosmotic transport to specific resistance of buffers bathing membrane. Male dog pericardium. Current strength 15 milliamperes. pH of buffers 7.36 to 7.44. Arrows and letters indicate order in which buffers were used. Mean values for each buffer plotted as white circles.

discontinued, since fluctuations in room temperature and heating effects with passage of current undoubtedly caused variations in viscosity greater than this.

The experimental arrangement has already been described (Fig. 1). Eight preliminary runs with inner electrode alternately cathode and anode were routinely made with each buffer to impregnate the membrane. Three pairs of runs were then made with 15 milliamperes current. The mean rates of liquid transport for these last runs are plotted against the specific resistances of the several buffers in Figs. 3 and 4.

In a simple homogeneous membrane of constant structure and composition the rate of electroendosmotic transport per unit of current is proportional to the specific resistance (σ) of the solution in the membrane pores.   Were the present membranes of this nature (see, however,[2]) the plots of volume transported against specific resistance should be straight lines, or should deviate appreciably from straight lines only through changes in the electrokinetic P.D.   How far the
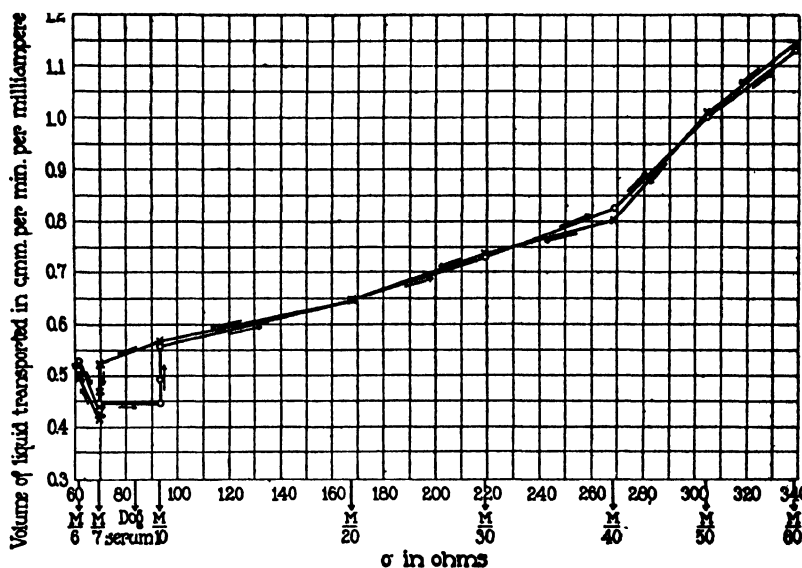
FIG. 4. Experiment 2.   Rate of transport and specific resistance of buffers. Female dog pericardium.   Current strength 15 milliamperes.   pH of buffers 7.37 to 7.41.   Arrows indicate order in which buffers were used. σ of M/7 and M/10 approximate only.

behavior of the present membranes differs from that of a simple inert membrane is shown by the discontinuity of the lines to the left-hand side of Figs. 3 and 4.

Two characteristics of the experimental curves are especially to be noted:

First, that the slope of the lines from $\sigma = 93$ ohms increases with increasing values of σ.   An increase in electrokinetic P.D. between solid and solution with decreasing salt content is a general phenomenon

[2] Mudd, S., *J. Gen. Physiol.*, 1924–25, vii, 389.

in the range of concentrations here used[3] and undoubtedly contributed to the upward trend of the lines with increasing values of σ. However, in consideration of what follows this explanation seems incomplete.

Second, the remarkable discontinuity of the curves in the region in which the conductivities and osmotic pressures of the buffers are close to those of the blood. Each of the points plotted is the mean value of six 6 minute runs. The buffer in the electrode vessel was changed after each run and other delays were incurred. The several points for any one buffer are therefore separated by intervals of from 1

TABLE I.

Experiment No.	Membrane.	State of animal.	Current strength.	Buffer.				σ of buffer.	Rate of transport per milliampere.	σ of serum of experimental animal.
			milli-amperes					*ohms*	*c.mm. per min.*	*ohms*
3	Dog mesentery.	Living.	25	(Phosphates + NaCl)			M/6.	61.83	0.36	83.54
	Dog mesentery.	"	25	"	"	"	M/7.	—	0.29	83.54
	Dog mesentery.	"	25	"	"	"	M/10.	—	0.39	83.54
4	Cat pericardium.	Dead.	15	"	"	"	M/6.	61.83	0.57	83.27
	Cat pericardium.	"	15	"	"	"	M/7.	69.68	0.49	83.27
	Cat pericardium.	"	15	"	"	"	M/10.	93.09	0.57	83.27

to several hours, and an overnight stay in buffer in the ice box usually intervened between the runs with the different buffers of $\sigma = 93$ ohms or less. The "hysteresis loops" at the left-hand side of Figs. 3 and 4 thus represent slow changes with time as well as with changing buffers in the rate of electroendosmotic transport across these pericardial membranes. These changes appear to have been completely

[3] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639, 655. Northrop, J. H., and Freund, J., *J. Gen. Physiol.*, 1923–24, vi, 603. Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 109, 395, 479; 1923–24, vi, 215.

reversible in Fig. 4, only partially so in Fig. 3.   On what alterations in structure and composition within the membrane they depended can only be guessed.   The equilibrium between membrane and environing medium was evidently very sensitive when the osmotic and electric conditions of the medium roughly approximated those of the blood.

The other two experiments affording data on rate of transport with the M/6, M/7, and M/10 phosphate and NaCl buffer mixtures show minimum values with M/7.   See Table I.

Throughout Experiments 1, 2, and 4, and only these afford data on this point, the mean rate of transport to the cathode was greater with inner than with outer electrode cathode when buffers M/6 to M/20 were used, and the rate of transport to the cathode was greater with outer than with inner electrode cathode when buffers M/30 to M/60 were used.   The differences were great at the extremes of the buffer series and gradually diminished to the transition point between M/20 and M/30.   With M/60, for instance, the mean rise of the meniscus toward the inner cathode was to the fall of the meniscus toward the outer cathode as 1 is to 1.6; with M/6, mean rise to inner cathode: mean fall to outer cathode:: 2.1:1.   These effects no doubt indicate that the membrane was polarized during the passage of current and that the polarization was somehow correlated with the conductivities of the perfusing buffers and with the arrangement of the membrane with respect to the inner and outer fluids.

Bethe and Toropoff[4] have demonstrated the polarization of diaphragms during passage of current.   Reversal with acid of the direction of electroendosmotic flow causes also reversal of the direction of polarization.   The relative disturbances in ion concentrations at the membrane surfaces are diminished by increasing salt concentrations in the perfusing solutions.

The Transport Rate with Serum.

The apparatus used for serum experiments (Fig. 5) was slightly modified from suggestions made by Dr. M. Kunitz.   A Zn-ZnSO$_4$ electrode connects through an L-way stop-cock with a salt bridge filled with 0.8 per cent NaCl solution.   A second L-way stop-cock near the other end of the bridge facilitates control of

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[4] Bethe, A., and Toropoff, T., *Z. physik. Chem.*, 1914, lxxxviii, 686; 1914–15, lxxxix, 597.

the several solutions.[5] The open-end vertical tubes are used for washing and filling. The rest of the arrangement is as already described.[2] Readings of the meniscus are made with the circuit closed. The mouth of the electrode vessel to which the membrane is applied is 6.5 mm. in internal diameter.

Serum was obtained by bleeding the animals, defibrinating, and centrifugating the blood; it contained sufficient hemoglobin to give a faint or deeper rose tint.
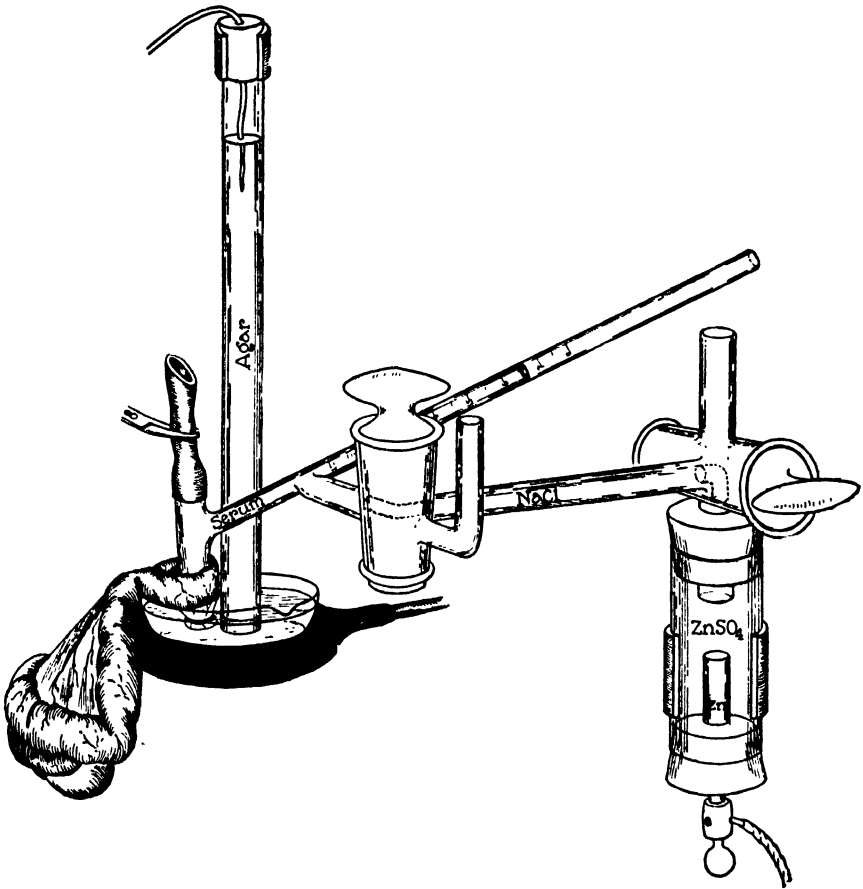
FIG. 5. Arrangement for study of electroendosmotic transport using whole blood serum and mesentery of living animal. See text.

[5] Replacement of this L-way cock by a T-way cock adapts the apparatus also to the determination of the H ion reversal points of membranous tissues. This form of the apparatus may be had from Arthur H. Thomas Company of Philadelphia.

The serum from animals fasted before use was clear.   If the animals had been newly fed the serum was turbid even after prolonged centrifugation, presumably due to lipemia.   Dark-field examination of such turbid serum showed myriad bright spherical objects ranging from the limit of visibility to droplets of a micron
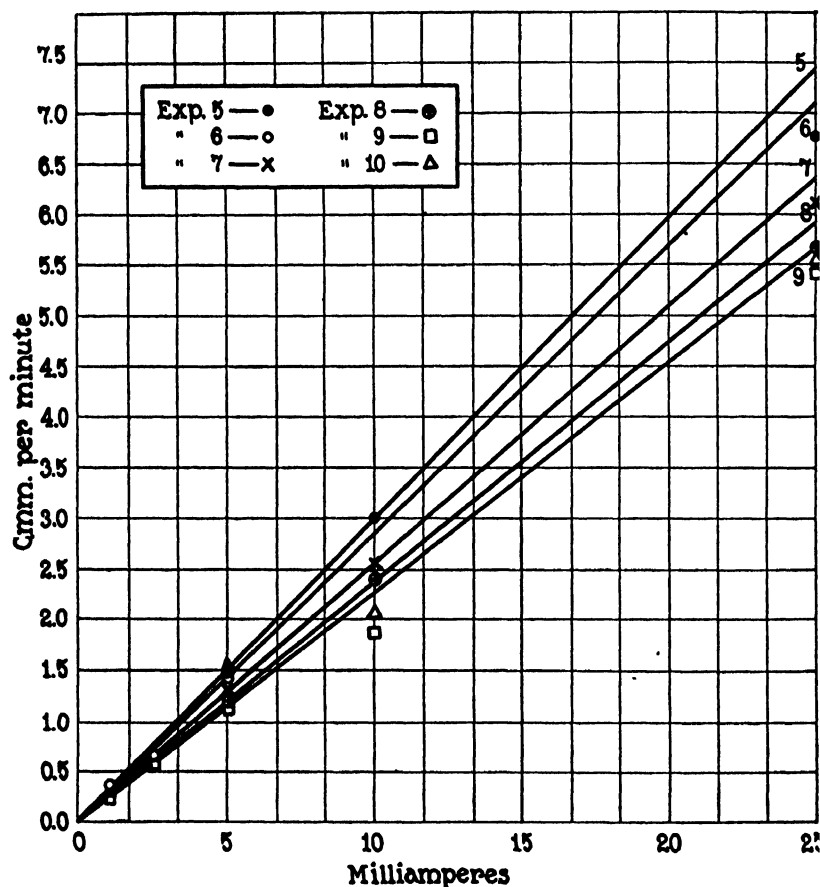
FIG. 6. Rates of transport of liquid plotted against current strengths.   Fluid bathing membranes, undiluted dog blood serum.   Experiment 5, living dog mesentery; Experiment 6, living dog mesentery; Experiment 7, dog mesentery, post mortem; Experiment 8, dog pleura, post mortem; Experiment 9, dog pericardium, post mortem; Experiment 10, dog pericardium, post mortem.

or more in diameter.   With the mesenteries of the living animals the fresh serum of another animal of the same species was used as perfusing fluid; precautions were taken against injury of the living mesentery.   With membranes used post mortem the serum of the animal from which the membrane came was used.

In general the points obtained with current strengths up to 10 milliamperes are seen to lie satisfactorily along straight lines passing through the origin (Fig. 6).

TABLE II.

Experiment No.	Serum.		Membrane.	State of animal.	Current strengths (in order used).	Rate of transport in c.mm. per min. per milliampere.
	From	State.				
					milli-amperes	
5	Dog B.	Clear.	Mesentery, Dog C.	Living.	10, 5, 25	0.30
6	" D.	"	" " E.	"	5, 2.5, 1	0.28
7	" C.	"	" " C.	Dead 23 hrs.	5, 10, 25	0.25
11	" A.	Turbid.	" " A.	" 3 "	25	0.27
9	" F.	Clear.	Pericardium, Dog F.	" 3 "	5, 2.5, 1 10, 25	0.23
11	" A.	Turbid.	" " A.	" 1½ "	25	0.23
8	" E.	Clear.	Pleura, Dog E.	" 18 "	5, 10, 25	0.24
12	" A.	Turbid.	" " A.	" 22 "	25	0.19*
13	Cat A.	Clear.	Mesentery, Cat. B.	Living.	25	0.22
14	" C.	Turbid.	" " D.	"	25	0.24
15	" C.	"	" " C.	Dead 7 hrs.	25	0.22
16	" B.	Clear.	Pericardium, Cat B.	" 4 "	25	0.26
17	" C.	Turbid.	" " C.	" 6 "	25	0.23
18	" B.	Clear.	Pleura, Cat B.	" 2 "	25	0.28
19	" D.	Slightly turbid.	" " D.	" 2 "	25	0.24
Average...						0.25

* The site of Experiment 12 seems to have been pleura from the anterior mediastinum rather than from the fibrous sheet between apex of pericardium and diaphragm ordinarily used. The section showed strands of atrophic thymus tissue and cysts filled with a coagulum between the pleural leaves.

Exceptions are the points plotted in triangles and the 10 milliampere point in Experiment 9. The points in triangles are, for reason not understood, so irregular that no attempt has been made to draw a line through them; they are not included in Table II.

All of the 25 milliampere values and the 10 milliampere point of Experiment 9 are lower than expectation. The disturbing factor is not known. The temperature of the serum bathing the membrane was raised a few degrees by the passage of the 10 and 25 milliampere currents, and this may have been of influence. Liquid flow may have become turbulent with the higher current strengths. However, the mean departure of observed values at 25 milliamperes from the straight lines amounted to only 5.4 per cent. The values of transport rate given in Table II for the experiments plotted in Fig. 6 are the slopes of the straight lines. For the experiments in which only 25 milliampere points are available the tabulated values for transport rate are probably about 5 per cent too low.

No certain correlation was detected between rate of transport and the thickness of the several membranes; this is in harmony with other electroendosmotic experiments.[6]

<div align="center">DISCUSSION.</div>

A number of animal membranes have been shown to be negatively charged relative to their environing medium when that medium is blood serum or other buffer of neutral or slightly alkaline reaction. The existence of this electric potential difference necessitates that the liquid in the membrane pores should tend to move toward the cathode when the membrane is traversed by an electric current. The rate of liquid flow has been shown to be proportional to the current strength and to amount when serum is used to 0.2 to 0.3 c. mm. per minute per milliampere.

The functional activity of glands[7] and muscles is known to be accompanied by electric current flow, and numerous other sources of current in the body are either known or may be confidently inferred from analogy with non-living systems. The suggestion has already been made[8] that the action current of glands might influence the

[6] von Smoluchowski, M., in Graetz, L., Handbuch der Elektrizität und des Magnetismus, Leipsic, 1914, ii, pt. 2, 380.

[7] Hermann, L., and Luchsinger, B., Arch. ges. Physiol., 1878, xvii, 310. Bayliss, W. M., and Bradford, J. R., J. Physiol., 1885, vi, p. xiii; 1886, vii, 217. Bradford, J. R., J. Physiol., 1887, viii, 86. Cannon, W. B., and Cattell, McK., Am. J. Physiol., 1916, xli, 39. Gesell, R., Am. J. Physiol., 1918–19, xlvii, 411.

[8] Mudd, S., and Mudd, E. B. H., J. Bact., 1924, ix, 163.

process of secretion. Consideration of the facts of the preceding paragraph, the writer believes, endows this possibility with a degree of probability amounting almost to certainty. For in such a system, in which liquid is being transported through capillary channels which are at the same time the site of an "action current" the electric current must at least modify if it does not control the liquid flow. Knowledge as to whether the electroendosmotic effect plays a major or minor part will have to await further study of the orientation and magnitude of the electric disturbances.

A number of the experiments here reported were performed by my technical assistant, Mr. Leo S. Hrdina.

SUMMARY.

The rate of electroendosmotic flow through dog and cat pericardia is found to be proportional to the current strength. The plots of current strengths against volumes of liquid transported in unit time are, in the better experiments, straight lines passing *through the origin;* the slopes of the lines are characteristic of the several systems.

Data on transport rate with buffers of different specific resistances showed the following phenomena:

1. Decrease of the observed transport rate to a minimum between σ values of 95 and 60 ohms.

2. Changes in the membrane markedly affecting transport rate, at conductivities and osmotic pressures close to those of the blood.

3. Polarization of the membrane during the passage of current.

The mean rate found for electroendosmotic transport across dog and cat serous membranes bathed in serum has been 0.19 to 0.30 (average, 0.25) c.mm. per minute per milliampere.[1] The best experiments with dog serum and the living mesenteries of dogs under ether gave a mean rate of 0.29 c.mm. per minute per milliampere.

These data, together with data from other sources, are believed to indicate a probability approaching certainty that electroendosmotic effects are a factor in glandular secretion.

MICROBIC VIRULENCE AND HOST SUSCEPTIBILITY IN PARATYPHOID-ENTERITIDIS INFECTION OF WHITE MICE.

IX. The Relationship of Dosage to Mortality Rate, Survival Time, and Cage Population.

By IDA W. PRITCHETT, Sc.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Early in the course of our studies on the epidemiology of mouse typhoid, Flexner[1] and Amoss,[2] discussing epidemics in general and experimental mouse typhoid epidemics in particular, drew attention to a possible relationship existing between dosage and the phenomenon of recurrent epidemic waves in an infected population. From the deaths occurring in his so called mouse village, where the spread of infection from cage to cage through the attendant who fed the mice and cleaned the cages was left to accident, Amoss was able to construct a typical epidemic curve, to which subsequent epidemic curves, following the addition of new mice to the infected population, closely conformed. Webster,[3] working with the same epidemic strain of mouse typhoid bacilli, demonstrated later that when virulence and population susceptibility were kept constant, dosage exerted a direct and quantitative effect upon mortality. By administering to each mouse a dose of 5,000,000 bacteria he was able to obtain, at will, mortality curves which could be superimposed upon Amoss' experimental epidemic curves. The same result was obtained indirectly by varying the number of infected mice in contact with normal unexposed animals. From these observations he concluded that "with a given susceptible mouse population and a certain strain of mouse typhoid bacilli

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[1] Flexner, S., *J. Exp. Med.*, 1922, xxxvi, 9.

[2] Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 45.

[3] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 269.

[in this case M. T. II] the sporadic and epidemic prevalences of mouse typhoid are determined by the spacial and quantitative distribution of the bacilli." He suggested, moreover, that the duration of life of normal contact mice added to an infected population may depend not only on their individual susceptibility, but on the degree of distribution of the bacilli in their environment.

In the experiments to be described, we have attempted to test these conclusions by determining in a mouse population infected with mouse typhoid the relationship between the fluctuating epidemic waves of mortality, and the available dosage, or number of mouse typhoid bacilli in the cage.

Method.

As a nucleus for these epidemics, there was arbitrarily chosen a population of ten mice of our Rockefeller Institute strain, each being injected *per os* with the usual standard dose of M. T. II (about 5,000,000 bacilli). This population was increased at a constant rate by the daily addition of two normal contact mice, also of our Institute strain. Two separate epidemics were thus studied at the same time, the mice of each epidemic being kept in a wire-topped zinc box measuring 5 × 7 × 10 inches. In each of the two boxes, as bedding for the mice, was placed a weighed quantity of very fine sawdust, which served also as a medium for the accumulation of bacteria excreted by the infected mice in the feces and urine. Preliminary experiments had shown that mouse typhoid bacilli, added in pure culture to such sawdust, were able to survive for periods of at least 3 or 4 days.

Besides controlling the population, we arbitrarily affected the dosage by periodically transferring the mice, in Box 1 every 2nd day and in Box 2 every 3rd day, to clean boxes containing fresh sawdust. Theoretically this might be expected to lead, in Box 1, to rapid and small fluctuations in the number of bacilli present, which would rise to a height every 2 days, and in Box 2 to less rapid and considerably greater fluctuations, reaching a peak every 3 days.

The following procedure was adopted for determining the number of mouse typhoid bacilli excreted by the mice and present in the sawdust. The whole volume of sawdust (50 gm.) was removed from the box and placed in a sterile bottle containing 500 cc. of beef infusion broth, after which the dirty boxes were subjected to steam sterilization. The bottle containing the sawdust-broth mixture was tightly corked and shaken in a shaking machine for 1 hour, in order to mix the material thoroughly with the broth and enable the masses of fecal material to soften. After shaking part of the supernatant broth was placed in large centrifuge tubes and whirled at low speed for a very short period—perhaps 30 seconds with the motor running—to throw down the heaviest particles which sometimes made it impossible to draw the uncentrifuged broth into the pipette. Dilution

plates were then poured in green dye agar, the dilutions of broth used for plating being 1:20, 1:200, 1:2000, and 1:20,000. These plates were incubated overnight at 37°C. and were counted the next morning.

For counting the plates a special technique was devised. First the total number of organisms present on the plate was counted in the usual manner. Frequently these all appeared to be typical for the mouse typhoid bacillus, but sometimes other organisms also developed, chiefly a colony resembling *Bacillus aerogenes*. When this happened it was necessary to estimate the number of mouse typhoid bacilli present. This was done by counting all the surface colonies, among which the colonies of M. T. II could be easily distinguished. The ratio of the mouse typhoid surface count to the total surface count was assumed to be the same as the ratio of the total typhoid count to the total count. From this ratio the total mouse typhoid count could be easily estimated. Having ascertained the number of mouse typhoid bacilli present in each cc. of the broth, it was necessary merely to multiply the result by 500 to obtain roughly the total number present, on this basis, in the whole 500 cc. of broth, and hence in the 50 gm. of sawdust taken from each box. The log of the value thus obtained was used in plotting a curve of the varying numbers of mouse typhoid bacilli present in each box and potentially available to the mice during the whole of the experimental period.

With the exception of the intervals at which the dosage was arbitrarily modified by changing the sawdust in the box, the mice in both epidemics received precisely the same treatment. They were fed daily on bread soaked in pasteurized Grade B milk, and two normal mice of our Rockefeller Institute strain were added daily as contacts. Each of these contact mice was marked with picric acid so that it could at any time be identified. At 9 o'clock each morning the boxes were gone over and all dead mice were recorded. Whenever possible, all these mice were autopsied and their organs cultured, but in about 73 per cent of the deaths occurring in each box this was rendered impossible through decomposition of the dead mice, or through the tendency of the living ones to eat their dead companions. As the experiment progressed, we hit upon the expedient of autopsying the mice as soon as they were found dead, and then returning them to the box in order that the conditions of the experiment might not be changed by removing this important source of infection from mouse to mouse.

During the second half of the experimental period, from about the middle of March on, difficulties were encountered in estimating the number of mouse typhoid bacilli present in the boxes, and it was eventually discovered that the original epidemic strain (M.T. II) had been almost entirely replaced by M.T. I (*Bacillus enteritidis*). This organism, antigenically dissimilar from our M.T. II strain, is known to be, to a mild degree, endemic among the breeding room mice of our Institute stock, and probably gained entrance to the boxes through the inadvertent inclusion of fecal carriers among the normal mice added as contacts.[4] The bacte-

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[4] During the period covered by these experiments—December 17, 1924, to June 5, 1925,—about 3700 mice of our Institute strain were given out from the breeding

rial counts for the second half of the experimental period represent, therefore, a mixture of M.T. I and M.T. II, the relative proportions of which are unknown.

EXPERIMENTAL.

On December 17, 1924, twenty albino mice of our Institute strain each received *per os*, by stomach tube, 0.5 cc. of a 1:100 dilution of an 18 hour broth culture of M.T. II, each dose containing about 5,000,000 bacilli. After inoculation, 10 of the mice were placed in each of two wire-topped zinc boxes, measuring 5 × 7 × 10 inches, to which had been previously added 50 gm. of very fine sawdust. Immediately thereafter, and on each day throughout the period of observation, from December 17, 1924, to June 4, 1925, inclusive, two normal mice of our Institute strain were added to each box as contacts. As already described, the sawdust was removed from each box, and its M.T. II content was determined at regular intervals, every 2nd day in Box 1 and every 3rd day in Box 2. This had the effect of periodically modifying the available dosage of mouse typhoid bacilli in each box.

The results of these two experiments are shown in Text-fig. 1, the curves for Box 1 being on the left, those for Box 2 on the right. The construction of the curve showing the fluctuations in the number of mouse typhoid bacilli present in the sawdust of the box has already been described. The mortality curve was constructed by plotting the total number of mice dying in each successive 2 day period in Box 1, and in each successive 3 day period in Box 2. The curve of the total cage population was made by plotting the average number of mice present in the box for the 2 day period in Box 1, or the 3 day period in Box 2. The curve of survival time was constructed by plotting the average survival time of the group of four contact mice added to Box 1 in each successive 2 day period, or the group of six contact mice added to Box 2 in each successive 3 day period. Following the scheme employed by Topley,[5] all mice dead in less than 3 days after being added to the box have been omitted from the curve of survival time as representing deaths probably due to fighting. Similarly, all mice which survived beyond the experimental period have been eliminated from this curve, their presence in the group being indicated by an arrow to show that if the experiment had been prolonged the average survival time for the group would have been somewhat greater than that recorded.

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room. In addition, about 300 mice of this strain were constantly kept on hand there as breeders. During this same period, 9 adult mice of our Institute strain died in the breeding room, from causes not associated with pregnancy, and were autopsied. Of these, 3 yielded colonies of M.T. I (*Bacillus enteritidis*) from the internal organs or feces. It will be seen from the figures that the incidence of M.T. I in our breeding room, as indicated by the deaths due to this organism, is very small indeed.

[5] Topley, W. W. C., *J. Hyg.*, 1920–21, xix, 350.

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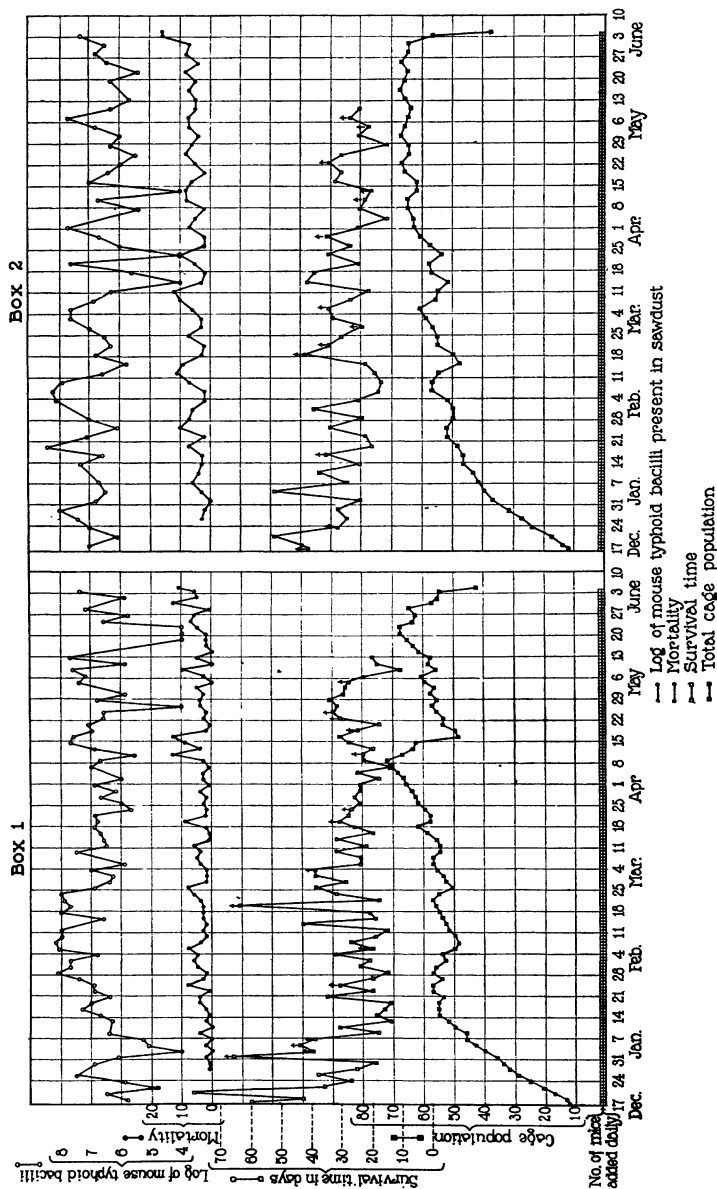
TEXT-FIG. 1.

A study of the curves in Text-fig. 1 will show that certain phenomena were common to both epidemics. In each box the mortality curve falls into successive wave-like fluctuations. The curve of total population, having a general upward trend throughout, likewise shows successive wave-like fluctuations. The same may be said of the curves of survival time and estimated dosage of M. T. II, both of which, however, show a slight downward trend.

Although in general the tendencies observable in the two boxes are the same, more careful analysis will show certain well marked differences. First may be mentioned the rapidity of the rhythm of the curves of survival time, mortality, and estimated dosage, which, in Box 1, is considerably greater than in Box 2. This, in all probability, is in some way a reflection of the intervals at which the sawdust was changed in the two boxes, the 2 day interval producing a more rapid rhythm in the cage number of bacilli than that caused by the 3 day interval. A further difference may be observed in the two boxes in the character of the mortality curves. In Box 1 the wave-like fluctuations in mortality, already mentioned, continue to the end of the experimental period. In Box 2, however, these waves become more and more shallow in the last 5 or 6 weeks of the experiment, the mortality rate assuming, in the end, a more or less constant level. The curves of total cage population may also be seen to differ somewhat in the two boxes. In Box 1 this curve falls to the end of the experimental period into well marked waves, the general tendency being upward, while in Box 2 it ceases to show these wave-like fluctuations during the last 5 or 6 weeks of the experiment and tends to assume a more or less constant level. It has already been noted that in both boxes, and especially in Box 2, the curve of total population shows, in general, an upward trend, while those of dosage and survival time exhibit a downward tendency. No explanation can be given at present for these findings.

The interrelationship of the curves of dosage, mortality, survival time, and total cage population may best be studied from those portions of the curves of Box 2, included in the period January 26 to March 15, shown in Text-fig. 2. This particular period is chosen because of the wide rhythm of these curves. On January 26 the mortality curve had reached a peak, and in the days immediately fol-

lowing the mortality decreased, reaching a low point February 4 to 7. During this decline in the epidemic wave the number of mouse typhoid bacilli present in the sawdust was steadily rising, reaching its peak on
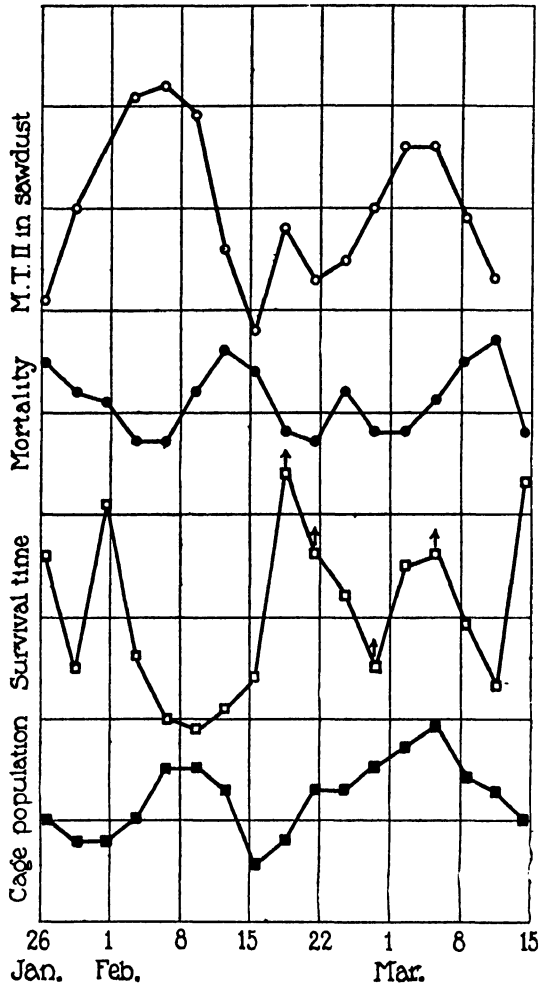
TEXT-FIG. 2.

February 7, at a time when the death rate was at a minimum. Immediately thereafter the mortality rate began to rise, reaching its peak 6 days after the peak in the bacterial count. The count continued to fall until February 16, after which there is recorded a slight

rise of very brief duration, which is, nevertheless, followed 6 days later by a small peak in the mortality curve. Again the bacterial count rose, reaching its maximum in the period March 3 to 6, and once more this peak is followed 6 days later by a peak in the mortality curve.

This recurrence of a 6 day interval between the period of maximum available dosage and the peak in the succeeding epidemic wave is, in our opinion, not a matter of chance. While it is shown most clearly in the period January 26 to March 15, it can be seen also in the two epidemic waves preceding January 26 (see Text-fig. 1, Box 2), so that in five successive epidemic waves, covering a period of 60 days, the same time relationship between high potential dosage and high mortality is found to hold good. Further significance is added to this fact, when we recall that in the many epidemics which we have initiated by inoculating *per os* the mice of our Institute strain with a known dose of mouse typhoid bacilli, the usual period of lag closely approximates this same interval. This is shown in the standard control curve constructed by Webster[6] for our Institute strain of mice, in which the period of lag is given as about 5 days, and in the similar curve reported for this same strain by Pritchett,[7] where the period of lag was found to be about 7 days.

A comparison of the curves of bacterial count and average survival time lends further emphasis to the relationship apparently existing between the number of bacteria present in the box and the mortality rate. It will be seen that as the bacterial count curve approaches its maximum, the survival time sharply declines, reaching its minimum at a point closely corresponding to the peak in the bacterial count. As the count falls again, the survival time rises sharply, to be again checked and brought down at points corresponding in general to peaks in the bacterial count.

The relationship of the population curve to the other curves, while not consistent throughout the experimental period, appears to be clear-cut in that portion of the chart shown in Text-fig. 2. It will be seen that the maxima in the total population curve closely correspond to the maxima in the bacterial count curve, while survival time

[6] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 231.

[7] Pritchett, I. W., *J. Exp. Med.*, 1925, xli, 195.

tends to decrease with crowding. It is possible that crowding may lower resistance in such a way that more rapid multiplication of bacteria becomes possible in the host, leading to a greater output.

The points which stand out from a study of Text-fig. 2 are, briefly, these:

1. Under the conditions described, there seems to be a definite time relationship between maximum available dosage and maximum mortality. This relationship is reflected in the curve of survival time, the fluctuations of which are inversely proportional to the number of mouse typhoid bacilli present in the cage.

2. There seems to be some relationship between the size of the total population and the number of mouse typhoid bacilli present in the cage.

Determination of Virulence.

On May 29 there was isolated from the sawdust of Box 2 a culture of the original epidemic strain, M.T. II. At this time, and for about a month preceding it, the mortality rate of the mice in Box 2 had ceased to show the well marked periodic fluctuations so characteristic of the earlier part of the epidemic, and was assuming a relatively constant level, while at the same time the population curve was also losing its previous wave-like character and remaining approximately constant. A test was performed with this recently isolated passage strain of M.T. II to determine whether it possessed a virulence superior to that of the original stock strain with which the epidemic had been initiated. 26 mice of our Institute strain received *per os*, with a stomach tube, 0.5 cc. of an 18 hour broth culture of the freshly isolated organism (4,350,000 bacilli), and at the same time 27 control mice of the same strain received 0.5 cc. of an 18 hour broth culture of the original stock culture (4,150,000 bacilli). Text-fig. 3 shows the mortality curves for these two sets of mice, together with the standard control curve constructed for this strain of mice last year, which so closely approximates the similar curve constructed for this strain by Webster. It will be seen that there is very little difference in the response of the mice to these two organisms, one recently isolated after repeated animal passage in the course of an epidemic, the other a stock strain which is kept in the ice box on agar slants, and has received no animal passage for more than 3 years. Such difference as exists is chiefly one of lag, a variation of 9 per cent in the final mortality rates being of little significance with such small numbers of mice.

Autopsy of Survivors.

It seemed to us desirable to study the mice remaining alive in each box at the end of the period of observation, in order to determine what

percentage of them showed the presence of living organisms in their tissues, or of specific agglutinins in their blood serum.

On June 5 all the mice remaining alive in both boxes were autopsied. Each mouse was etherized, and while it was still breathing the abdomen and thorax were opened, care being taken not to sever any of the large blood vessels. With a small gauge needle and a 1 cc. Luer syringe as much blood as possible was removed from the right ventricle, while the heart continued to beat. A drop of this blood was placed on a 0.1 per cent glucose agar plate divided into quarters, and spread
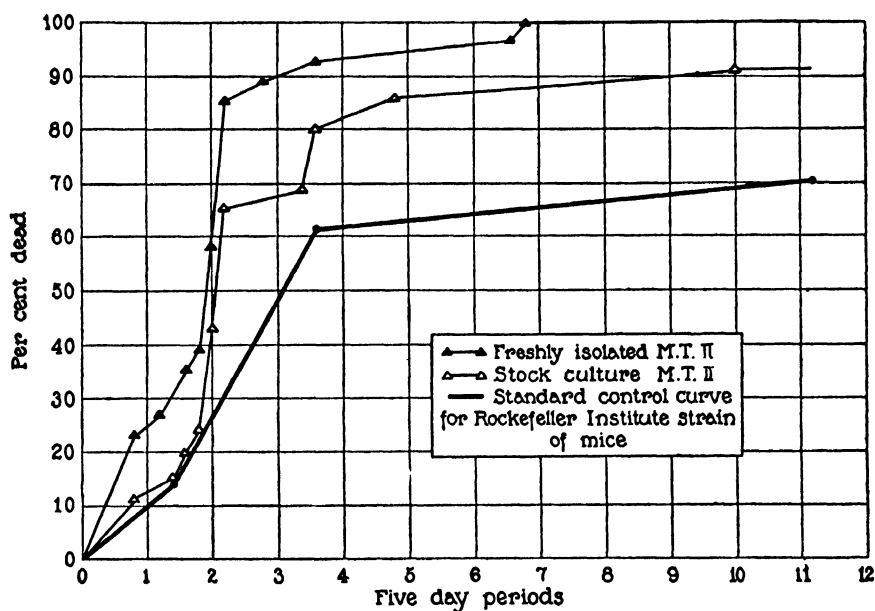
TEXT-FIG. 3.

with a loop, the rest of the blood being placed in a slanting position in a sterile, plugged Wassermann tube and allowed to clot. Cultures were also made on the agar plate from the spleen, gall bladder, and feces, and the gross appearance of all these organs, and of the liver, was noted. Only 43 mice from Box 1 and 37 mice from Box 2 came to autopsy on June 5, because of the large number of deaths in both boxes due to the excessively high temperature prevailing in New York City during that week. The same effect was observed among the normal mice in our breeding room, large numbers of them succumbing to the heat during this period.

The pathological, serological, and cultural findings from all these mice are given in Tables I and II.

It will be seen from a study of Tables I and II that the vast majority

TABLE I.

Box 1.		Gross pathology.				Suspicious colonies.				Mouse typhoid bacilli. Serological types isolated.				Titre blood agglutinins.	
Mouse.	Days in box.	Liver.	Spleen.	Gall bladder.	Feces.	Heart's blood.	Spleen.	Feces.	Gall bladder.	Heart's blood.	Spleen.	Feces.	Gall bladder.	M.T. I.	M.T. II.
16	155	Normal.	±	+	±	—	+	+	+		I	—	—	—	—
17	154	"	Normal.	Normal.	Normal.	—	—	+	—			—		640	—
19	152	"	"	"	"	—	+?	+	—			—		40	—
39	132	+	+	+	±	—	+	+?	+		I	—	I	—	—
51	119	Normal.	Normal.	Normal.	Normal.	—	—	+	—		I	—		—	—
66	105	+	"	±	+?	—	+	+	—		I	—		—	—
78	93	+	"	Normal.	Normal.	—	—	—	+		I	—		—	—
93	78	Normal.	"	"	"	—	—	+?	—			—		—	—
98	73	±	±	+?	+?	—	—	+	+			—		—	—
116	55	Normal.	Normal.	Normal.	Normal.	—	—	—	—	—	—	—	—	—	—
123	48	±	"	"	"	+	+	+?	+	I	I	—	—	160	—
127	44	Normal.	"	"	"	+	+	—	—	I	I	—	I	—	—
129	42	"	"	"	"	+	+	+	—	I	I	—		—	—
135	36	"	"	"	"	—	—	+?	—			—		—	—
138	33	"	"	"	"	—	+?	+?	+?			—		—	—
139	32	"	"	"	"	—	—	+?	—			—		—	—
143	28	"	"	"	"	+	+	+?	—	I	I	—	I	—	—
149 A	22	"	"	"	"	+	+	+?	—	I		—		—	—
149 B	22	"	"	"	"	—	+	+	—			—		—	—
150	21	"	"	±	"	—	—	+?	+			—		—	—
151	20	+	+	Normal.	"	+	+	+	+	I	I	—	I	—	—
153 A	18	Normal.	Normal.	"	"	+	+	—	+	I	I	I	I	—	—
153 B	18	"	"	"	"	—	—	—	+		I	—	I	—	—
154	17	"	"	"	"	—	—	+	—			—		—	—

	No.														
156	15	Normal.	Normal.	Normal.	Normal.	+	−	+	+	−	−	1	−	1	1
157 A	14	"	"	"	"	−	−	−	−	−	−	−	−	−	−
157 B	14	"	"	"	"	+	*+	+	*+	+	−	1	−	1	1
159 A	12	"	"	"	"	−	+?	−	−	−	−	−	1	−	−
159 B	12	"	"	"	"	−	−	−	−	−	−	−	1	−	−
160	11	"	+?	"	"	−	+?	−	−	−	1	1	1	1	1
161 A	10	"	Normal.	"	"	−	+	−	−	+	−	−	−	−	−
161 B	10	"	+?	"	"	−	+	+	+	−	−	−	−	−	−
162	9	"	Normal.	"	"	+	−	−	−	−	1	1	1	1	1
163	8	"	"	"	"	−	+	+	−	−	−	−	−	−	−
164	7	"	"	"	"	−	−	−	−	−	−	−	−	−	−
165 A	6	"	"	"	"	−	+	−	−	+	−	−	−	−	−
165 B	6	"	"	"	"	+	+	+	−	−	−	1	−	−	−
166 A	5	"	"	"	"	−	−	+	*+	−	−	−	−	−	−
166 B	5	"	"	"	"	−	+	−	−	*+	−	−	−	−	−
167	4	"	"	"	"	−	+	−	−	−	−	−	−	−	−
169	2	"	"	"	"	−	+	−	−	−	−	−	−	−	−
170 A	1	"	"	"	"	−	−	−	−	−	−	−	−	−	−
170 B	1	"	"	"	"	−	+?	−	−	−	−	−	−	−	−

* Mucoid colonies only.

TABLE II.

Box 2.		Gross pathology.				Suspicious colonies				Mouse typhoid bacilli. Serological types isolated.				Titre blood agglutinins.	
Mouse.	Days in box.	Liver.	Spleen.	Gall bladder.	Feces.	Heart's blood.	Spleen.	Feces.	Gall bladder.	Heart's blood.	Spleen.	Feces.	Gall bladder.	M.T.I.	M.T.II.
Inf.*	170	Normal.	Normal.	Normal.	Normal.	—	—	+?	+?			—	—	—	—
7	164	"	"	"	"	—	—	+?	—					—	—
25	146	"	"	"	"	—	+	+?	—		II			—	—
30	141	"	"	"	"	—	—	+?	—					—	—
63	108	"	"	"	"	—	—	+?	—					—	—
68	103	"	"	"	"	—	—	+?	—					—	—
74	97	"	"	"	"	—	+	+?	—		I	I		—	—
78	93	"	"	"	"	—	—	+	—					—	—
79	92	"	"	±	"	—	+?	—	—		II			—	—
103	68	"	"	Normal.	"	—	—	+?	—					—	—
114	57	"	"	"	"	—	—	+	—					—	—
117	54	"	"	"	"	—	—	+?	—					—	—
128	43	"	"	"	"	—	—	—	—					—	—
139	32	"	"	"	"	—	+	+?	—					—	—
140	31	"	"	"	"	+	+	+†	+	I	I		I	—	—
142	29	"	"	"	"	—	+†	+	+†	I	I	I	I	—	—
143	28	"	"	"	"	+†	+†	—	—	I	I		I	—	—
148	23	"	"	"	"	+†	—	+?	—					—	—
149 A	22	"	"	"	"	—	—	+?	—					—	—
149 B	22	"	"	"	"	—	—	—	—			I		—	—
154	17	"	"	"	"	+	+	+?	+	I	I		I	—	—
156	15	"	"	"	"	—	—	+?	—			I		—	—
158 A	13	"	"	"	"	—	—	+?	—			I		—	—
158 B	13	"	"	"	"	—	—	+?	—			—		—	—

Mouse	No.						S1	S2	S3	S4						
161 A	10	Normal.	Normal.	Normal.	Normal.	Normal.	−	−	+	−	I	I	−	−	−	−
161 B	10	"	"	"	"	"	+	+	+	−	−	I	−	I	−	−
162 A	9	"	"	"	"	"	−	+	+	+	−	−	−	−	−	−
162 B	9	"	"	"	"	"	−	−	−	−	−	−	−	−	−	−
163 A	8	"	"	"	"	"	−	−	+?	−	−	−	−	−	−	−
163 B	8	"	+?	"	"	"	−	−	+	+?	−	−	−	−	−	−
164	7	"	Normal.	"	"	"	−	−	+?	+	−	−	−	−	−	−
165	6	"	"	"	"	"	−	−	−	−	−	−	−	−	−	−
166 A	5	"	"	"	"	"	−	−	+	−	−	−	−	−	−	−
166 B	5	"	"	"	"	"	−	−	−	−	−	−	−	−	−	−
167 A	4	"	"	"	"	"	−	−	+	−	−	I	−	−	−	−
167 B	4	"	"	"	"	"	−	+	+	−	−	−	−	I	−	−
169	2	"	"	"	"	"	−	−	+	+	−	−	−	−	−	−

* One of the mice originally infected *per os* by stomach tube.
† Mucoid colonies also found.

of the mice in both boxes, coming to autopsy on June 5, showed no gross indications of having been infected with the bacillus of mouse typhoid. As is indicated by plus marks, an occasional liver showed slight scarring, the spleen was sometimes slightly swollen or scarred, a few gall bladders were whitish in appearance, and in a very few mice the feces were perhaps faintly tinged with blood. Nevertheless, mouse typhoid bacilli, identified by agglutination tests, were isolated from the heart's blood, feces, and internal organs in a fairly high percentage of cases (Table III).

The predominating organism isolated from these mice was not, however, the organism with which the epidemic was initiated (M.T. II), but rather the M.T. I which probably gained entrance to the boxes,

TABLE III.

	Surviving mice positive for mouse typhoid bacilli.								Total positive.	
	Heart's blood.		Spleen.		Feces.		Gall bladder.			
	M.T. I.	M.T. II.	M.T. I.	M.T. II.	M.T. I.	M.T. II.	M.T. I.	M.T II.	M.T. I.	M.T. II.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Box 1....................	21	0	37	0	9	0	19	0	42	0
Box 2....................	11	0	22	5	8	0	11	0	27	5

as already indicated, through the presence of healthy carriers among the mice added as contacts. In Box 1 the original M.T. II had apparently been completely eliminated, while in Box 2 it was found in only 5 per cent of the mice examined, each time in the spleen. Yet it was probably still present in small numbers even at this time, in both boxes, since Type II organisms were found, together with Type I, in Mouse 138, Box 1, which died June 2, after living 30 days in the box, while both types were recovered from the sawdust of Box 1 as late as April 13. In Box 2 both types were recovered from Mouse 157, dying June 5, after living 14 days in the box, and Type II, together with Type I was recovered from the sawdust of the box as late as May 29.

DISCUSSION.

Topley, in his early studies of mouse typhoid epidemics, attributed the rise and fall and periodic recurrence of epidemic waves to fluctuations in the virulence of the microbic incitant.[8]   On this basis also he explained the fact that the duration of life of additions made to a population in the early stages of an epidemic was considerably shorter than when they were introduced after the period of maximum mortality had passed.[5]   Webster, on the contrary, could find no significant fluctuations in the pathogenicity of mouse typhoid bacilli,[9] and from the evidence gained through an experimental analysis of virulence, microbic dosage, and host susceptibility believed that the phenomena of recurrent epidemic waves arise from fluctuations in the two latter factors.

The experiments described in this paper were planned to test these opposing points of view under conditions as natural and as carefully controlled as may be.   Shortly before they were begun, Topley and his coworkers published the results of a study of the number of mouse typhoid bacilli excreted from day to day by each member of an infected mouse population.   They found that the excretion rate of the various individuals composing the group rose simultaneously before a rise in mortality rate, and declined at the time of its fall.[10]   In a more recent paper on mouse pasteurellosis, Greenwood and Topley[11] showed that when larger numbers of normal mice were added daily to an infected population the resulting mortality curve tended to assume a high general level, with relatively slight fluctuations and minimal interepidemic periods.   As the number of daily additions was decreased, the height and duration of the successive epidemic waves was increased, and the interepidemic periods became well marked and of considerable extent.   In our experiments, the mortality curve with slight and rapid fluctuations occurred in the community where the dosage was affected arbitrarily by changing the sawdust at short intervals, while in the community whose mortality curve showed ample waves and well

[8] Topley, W. W. C., *Lancet*, 1919, ii, 1.

[9] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 781.

[10] Topley, W. W. C., Ayrton, J., and Lewis, E. R., *J. Hyg.*, 1924–25, xxiii, 223.

[11] Greenwood, M., and Topley, W. W. C., *J. Hyg.*, 1925, xxiv, 45.

marked interepidemic periods the interval at which dosage was arbitrarily modified was longer. Hence by affecting the rate of immigration, or by varying the available dosage directly, one can modify at will the character of the epidemic waves and the interepidemic period. Thus our observations agree with the recent ones of Topley and his coworkers and, taken together with them, constitute strong evidence of the view put forward by Webster,[3] concerning the importance of dosage.

One further point in the resistance of the survivors of an epidemic deserves emphasis at this time. Webster has pointed out that a certain percentage of mice, given an infecting dose of mouse typhoid bacilli *per os*, shows no signs of infection,[6] and he later showed that when survivors were exposed a second time to a similar infection it was this so called natural resistance as much as any specific acquired immunity which again protected them.[12] Topley, however, in his examination of survivors, has been able to cultivate the bacilli from the internal organs in most instances, and he inclines to the view that specific acquired immunity, and perhaps a type of depression immunity similar to that studied by Lange[13] and by Morgenroth,[14] are responsible for the enduring resistance. The survivors in our own experimental series are of interest, when examined from this point of view, since they show a large percentage of naturally resistant individuals free from all signs of infection.

SUMMARY.

Epidemics of mouse typhoid set up among the Rockefeller Institute strain of mice were studied over a period of 6 months. During this time the relationship of cage number of mouse typhoid bacilli to mortality, total population, and survival time was determined. A single virulence titration of the epidemic strain was made, and at the end of the experiment all survivors were examined for evidence of infection. The following conclusions may be drawn for the data here presented.

[12] Webster, L. T., *J. Exp. Med.*, 1924, xxxix, 129.

[13] Lange, B., *Z. Hyg. u. Infectionskrankh.*, 1921, xciv, 135; 1924, cii, 224.

[14] Morgenroth, J., Biberstein, H., and Schnitzer, R., *Deutsch. med. Woch.*, 1920, xlvi, 337.

1. The available dosage of mouse typhoid bacilli varied directly with the mortality (plus a time constant of 6 to 8 days) and inversely with the survival time.

2. The virulence of the epidemic strain appeared to be practically the same as that of the original stock culture.

3. About 53.5 per cent of the survivors of one epidemic and 68 per cent of those in the other showed, at the end of the experiment, no signs of infection; the others had either specific blood agglutinins, or living bacteria in their heart's blood, spleen, feces, or gall bladder.

4. During the course of the epidemic, the original infecting strain (mouse typhoid Type II—*Bacillus pestis caviæ*) was almost entirely replaced by an antigenically dissimilar strain (mouse typhoid Type I—*Bacillus enteritidis*), probably introduced through the inadvertent inclusion of fecal carriers among the normal mice added as contacts.

MICROBIC VIRULENCE AND HOST SUSCEPTIBILITY IN PARATYPHOID-ENTERITIDIS INFECTION OF WHITE MICE.

X. The Relative Susceptibility of Different Strains of Mice to Per Os Infection with the Type II Bacillus of Mouse Typhoid (Bacillus Pestis Caviæ).

Further Studies.

By IDA W. PRITCHETT, Sc.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In a previous paper[1] we have compared the relative susceptibility of five different strains of mice to *per os* inoculation with a standard dose of mouse typhoid bacilli, Type II (about 5,000,000 organisms). Experiments have been continued through another year with four of these strains, two white and two colored, in which the following numbers of each strain were used.

Bagg mice (albino)	576
Lathrop mice (black)	631
Little mice (dilute brown)	313
Rockefeller Institute mice (albino)	580
Total	2100

The histories of these mice, as far as they are known to us, have already been recorded.[1]

EXPERIMENTAL.

As in the preceding year, monthly tests were run with all these strains of mice, beginning in October, 1924, so that the entire series

[1] Pritchett, I. W., *J. Exp. Med.*, 1925, xli, 195.

of experiments, for both years, covers a period of 24 consecutive months, from October, 1923, to September, 1925 inclusive.   An effort was made to have at least 50 mice of each strain for each monthly test, but in some instances the number fell below this standard, especially with the Little mice.   The total number of mice, therefore, was only 2100, as compared to a total of 2540 mice of these four strains in the previous year.   All strains were strictly strain-inbred.

The routine procedure in starting one of these monthly tests was as follows:

The mice of all strains were brought from the breeding room and assembled on the day before inoculation, each mouse in a separate battery jar partly filled with shavings.   The next morning each mouse received *per os*, with a stomach tube, 0.5 cc. of an 18 hour broth culture of M.T. II, diluted 1:100, this dose containing usually about 5,000,000 organisms.   All animals were fed directly after the administration of the infecting dose with the usual daily diet of bread soaked in pasteurized Grade B milk.   At about 9 o'clock each morning the deaths for the preceding 24 hours were recorded.   No autopsies were performed on any of these mice, since our previous experience had indicated that only a very small percentage of our deaths was due to non-specific causes.   All experiments were allowed to run 8 weeks.

In Text-figs. 1 to 4 are shown all the twelve curves plotted for each of the four strains of mice, in the course of the 12 month period.   These charts may be compared with the corresponding charts in the paper already referred to,[1] where the curves for the previous 12 month period are shown.   Text-fig. 1 gives the twelve curves for the Rockefeller Institute mice.   It will be seen from this chart, as well as from Text-figs. 2, 3, and 4, which show the curves for the Bagg, Lathrop, and Little mice respectively, that the scatter in the various monthly curves is rather more than that recorded for these same strains in the preceding year.   The Lathrop mice, however (Text-fig. 3), remained the most constant in their response to the infection, showing about the same degree of scatter that they showed in the previous year.   A comparison of the scatter shown by the different strains for the 2 years is given in Table I.

Although the degree of scatter is slightly greater this year than last, the various strains retain the same relative standing with respect to each other.   The Lathrops and Littles, both colored strains, show still a greater degree of constancy in their response to the infection, as
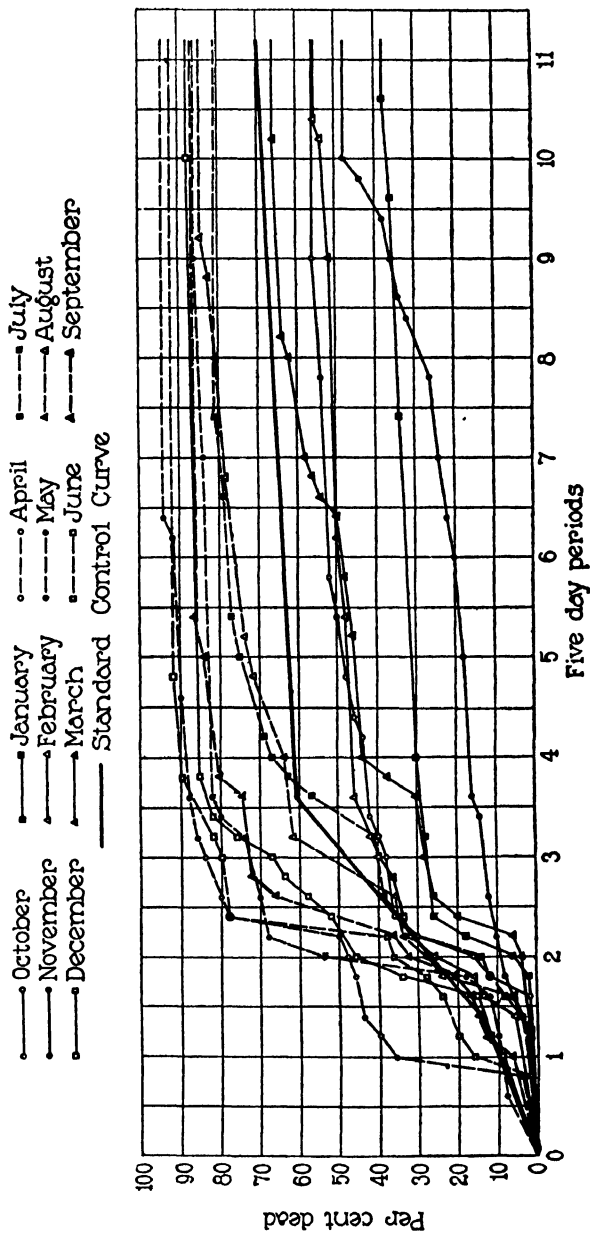
TEXT-FIG. 1. Rockefeller Institute mice.
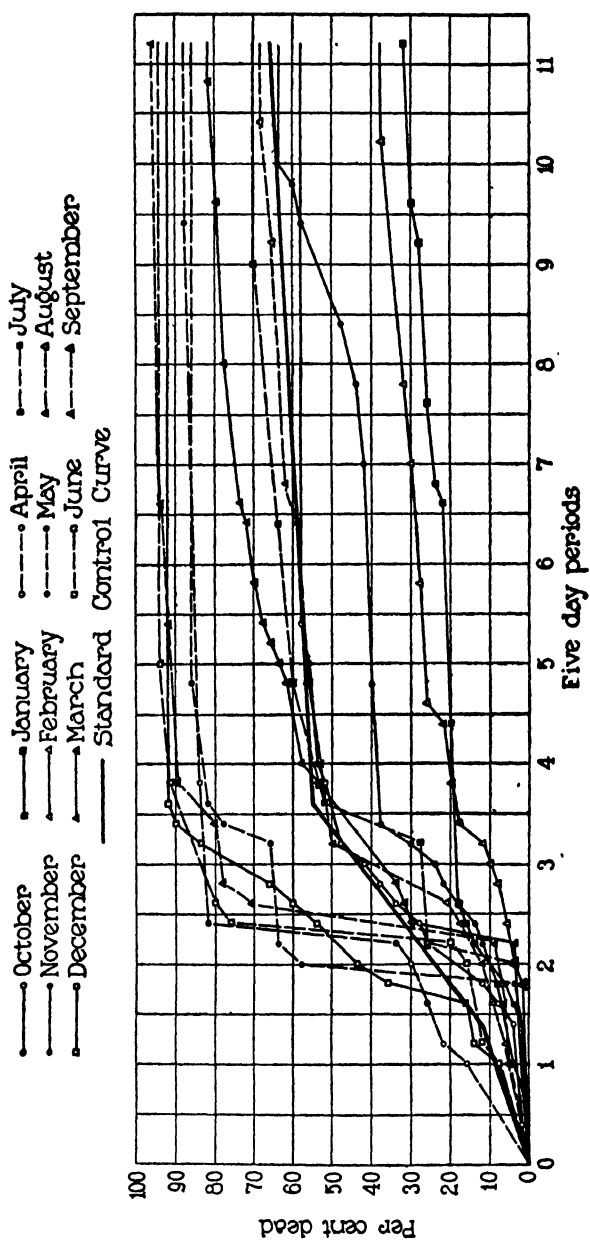
TEXT-FIG 2. Bagg mice.
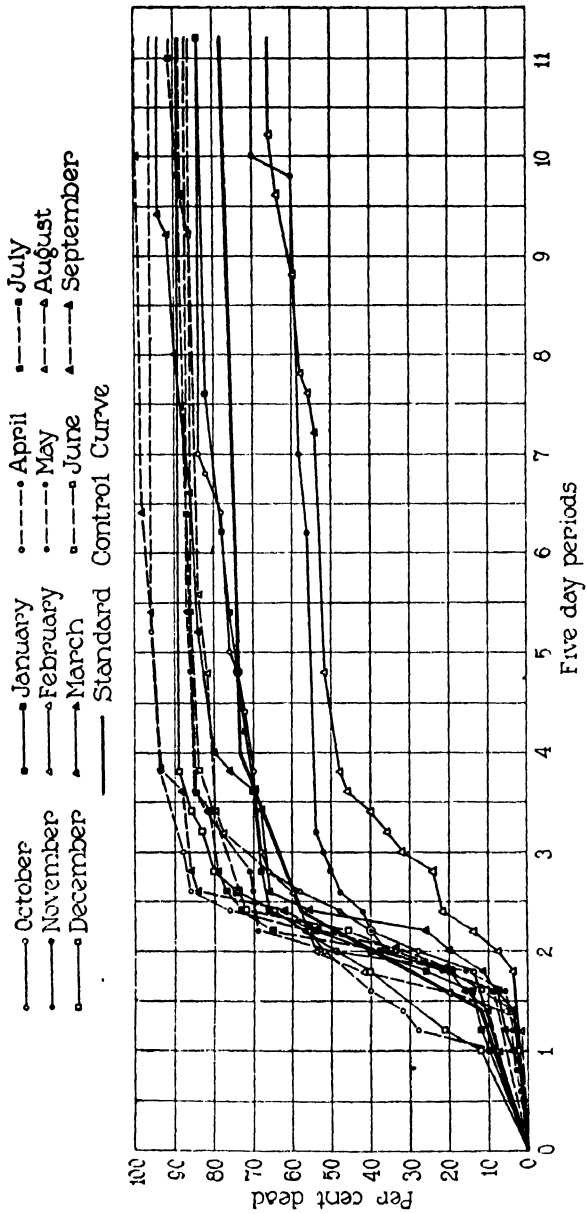
TEXT-FIG. 3.   Lathrop mice.
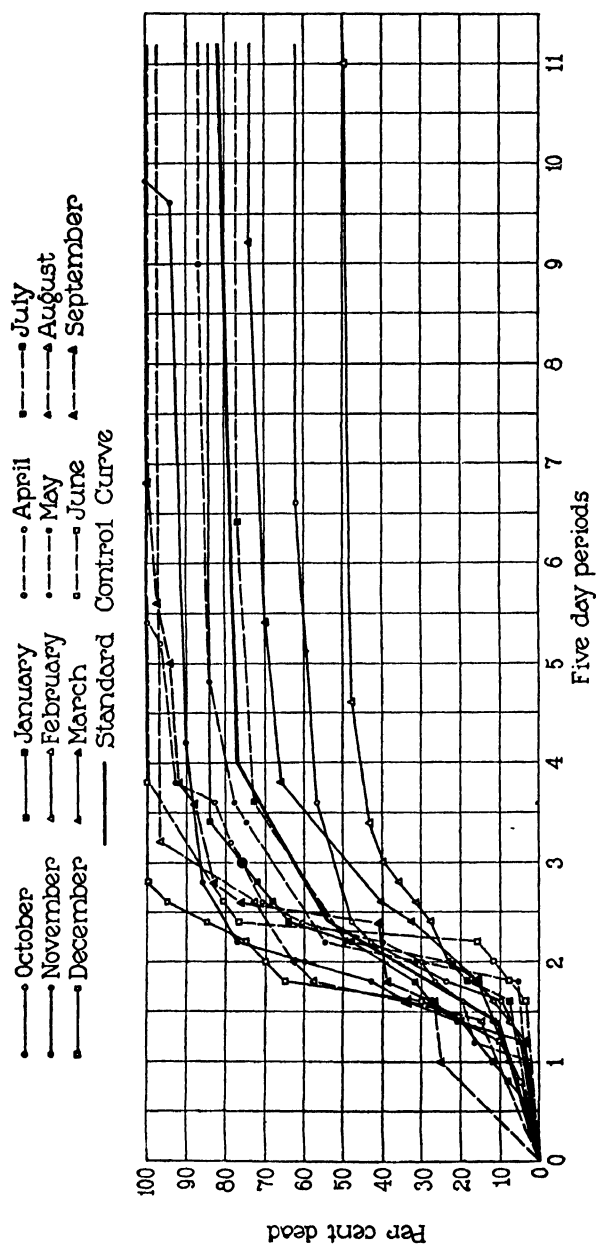
TEXT-FIG. 4. Little mice.

indicated by the degree of scatter in their curves, than do the albino strains. The seasonal variation in the susceptibility of the different strains, which is the source of the scatter in the curves, will be discussed in another paper.[2]

In Text-fig. 5 are shown average curves constructed for each strain of mice from all the curves for that strain in the course of the 12 month period, together with the standard control curve obtained last year for the Rockefeller Institute strain. Reference to the earlier paper,[1] and to Text-fig. 6 of the present paper will show that these average curves closely approximate, both in height and pitch, the similar curves obtained for these four strains of mice last year. Again the average mortality is lower for the albino strains (Bagg and Rockefeller Institute), the two dark strains (Lathrop and Little) showing a final

TABLE I.

Strain.	Difference separating lowest and highest mortality rates.	
	per cent, 1923–24	*per cent, 1924–25*
Lathrop mice (black).................................	30	34
Little " (dilute brown)...........................	34	50
Institute " (albino)................................	50	56
Bagg " (albino)................................	55	65

mortality about 13 per cent above that of their white companions. As was also observed last year, the pitch of the curves for the colored strains is steeper than that of the curves for the white strains. In short, for all strains much the same relative susceptibility may be seen to exist that was recorded in the previous 12 month period.

In Text-fig. 6 are shown the average curves constructed for each of the four strains of mice this year and last. It will be seen how closely the curves obtained in the second year approximate those for the preceding 12 month period, the final mortality rate for all strains being slightly higher.

In Text-fig. 7 are shown standard control curves constructed for each strain from all the mice used in these experiments to date. The standard control curve for the Rockefeller Institute strain has been

[2] Pritchett, I. W., *J. Exp. Med.*, 1926, xliii, 173.
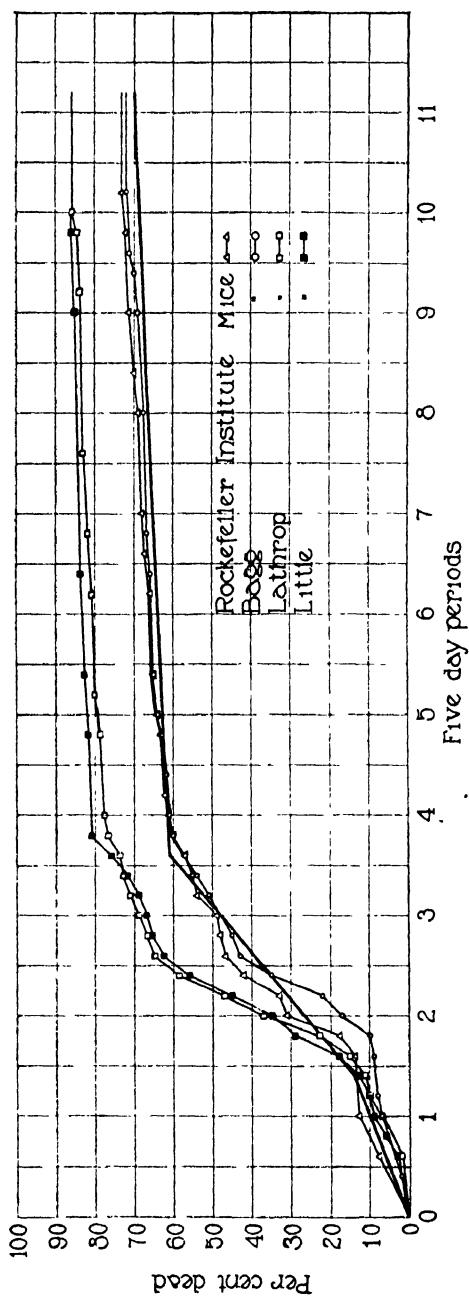
TEXT-FIG. 5.
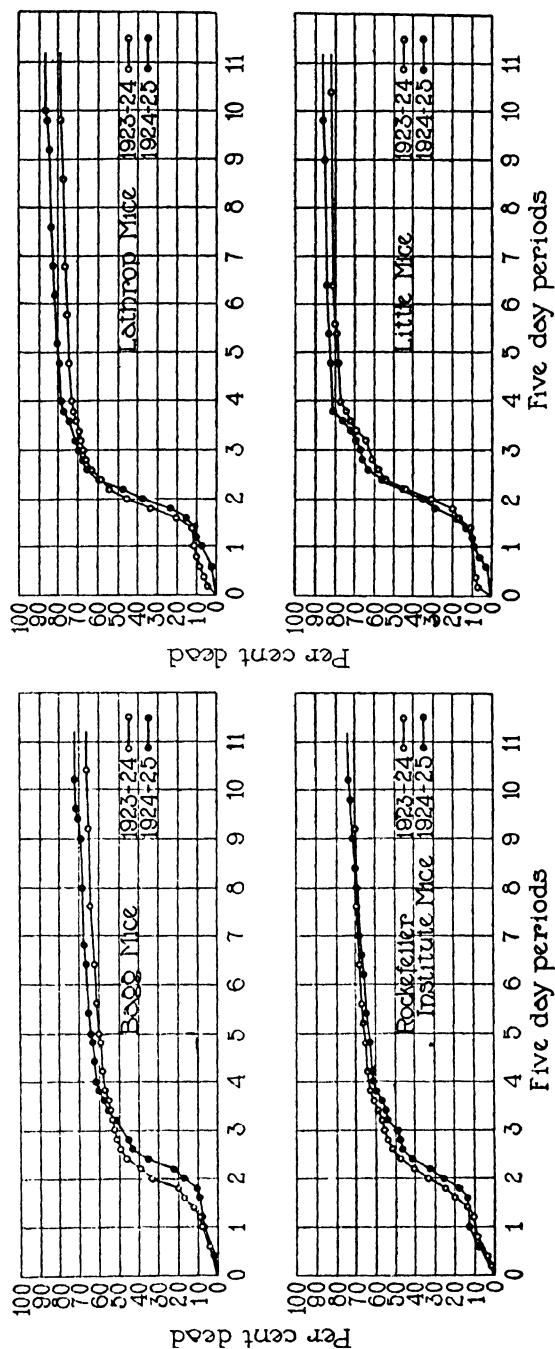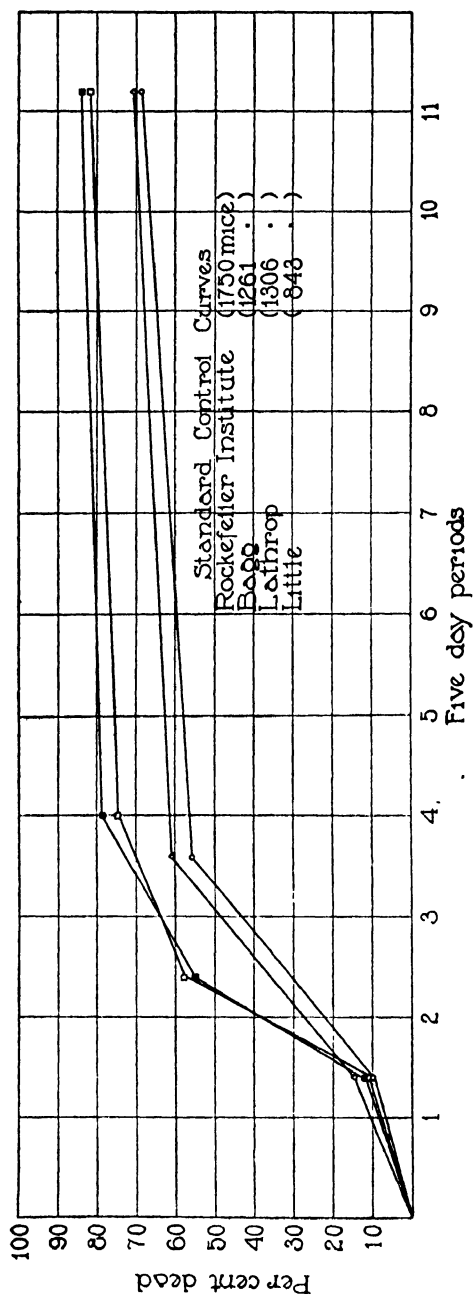
TEXT-FIG. 7.

constructed by averaging the two curves obtained for this strain in the past 2 years, together with the standard control curve originally constructed for this strain by Webster,[3] which the two later curves so closely approximate. The various standard control curves given in Text-fig. 7 are based on the following numbers of mice.

Bagg (albino)... 1261 mice.
Lathrop (black).. 1306 "
Little (dilute brown)................................... 843 "
Rockefeller Institute (albino).. .... 1750 "

The large numbers of mice of known age and parentage employed in these experiments; the repeated monthly tests over a period of 2 years, with all conditions as nearly as possible the same; the characteristic mortality curve of each strain, varying from month to month in a more or less predictable manner, and yet retaining a constant mean; the same consistent differences between the races—all these things show clearly that these strains of mice inherit, from generation to generation, a definite amount of resistance, affected by external influences, but under constant conditions varying among different individuals about a mean which is characteristic of the race.

Further evidence for this belief has been brought forward by Webster, who has shown that selective breeding of the most resistant or most susceptible individuals in a given race transmits a greater or less resistance to succeeding generations.[3,4]

SUMMARY.

Four separate strains of mice have been tested for their relative susceptibility to *per os* infection with the Type II bacillus of mouse typhoid (*Bacillus pestis caviæ*), 300 to 600 individuals of each strain having been employed in the course of 12 months. In confirmation of a previous paper, clear-cut differences in the susceptibility of these strains have been shown to exist. In general, the colored strains were distinctly less resistant than the albino strains.

[3] Webster, L. T., *J. Exp. Med.*, 1924, xxxix, 879.
[4] Webster, L. T., *J. Exp. Med.*, 1925, xlii, 1.

MICROBIC VIRULENCE AND HOST SUSCEPTIBILITY IN PARATYPHOID-ENTERITIDIS INFECTION OF WHITE MICE.

XI. Seasonal Variation in the Susceptibility of Different Strains of Mice to per Os Infection with the Type II Bacillus of Mouse Typhoid (Bacillus pestis caviæ).

Further Studies.

By IDA W. PRITCHETT, Sc.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In a recently published paper[1] we have drawn attention to the fact that a marked seasonal variation in susceptibility to infection with the bacillus of mouse typhoid has been observed with five unrelated strains of mice, all fed on the same food and subjected to the same living conditions. The experiments previously reported were begun in October, 1923, and those to be discussed now, performed with four of the five strains, followed directly the ones of the previous year. Thus we have a record of the response of these four strains of mice to a standard dose of the bacillus of mouse typhoid from October, 1923, to September, 1925, inclusive, the only gaps in the record being for the Lathrop strain in November, and for the Little strain in October and November of 1923.

EXPERIMENTAL.

The material presented here is the same as that employed in another paper on the relative resistance of different strains of mice to mouse typhoid,[2] the Bagg, Lathrop, Little, and Rockefeller Institute strains

[1] Pritchett, I. W., J. Exp. Med., 1925, xli, 209.
[2] Pritchett, I. W., J. Exp. Med., 1926, xliii, 161.

being used for these tests.   The histories of these strains are given elsewhere.[3]

As in the preceding year, routine monthly tests were made with all four strains of mice for the period from October to September inclusive.   The procedure employed in starting one of these monthly tests was the same as that used in the earlier work.   On the day before inoculation with the mouse typhoid bacillus, each mouse was placed in a separate battery jar partly filled with shavings.   The next morning a dilution of 1:100 of an 18 hour broth culture of M. T. II was made in bouillon, from which three dilution plates were poured for the purpose of counting the organisms present.   Each mouse then received by stomach tube 0.5 cc. of this 1:100 dilution of the original culture, containing on an average about 5,000,000 mouse typhoid bacilli.   The whole series of mice was fed directly after the inoculation with the usual daily diet of bread soaked in pasteurized Grade B milk.   Each test was allowed to run for 8 weeks, during which time the deaths were recorded daily and mortality curves were plotted.

In Text-fig. 1 are shown the results of the twelve monthly tests. The charts are arranged in chronological order, from above downward, and in each chart is included in addition to the curves for the four strains of mice, the standard control curve constructed last year for our Institute albino strain, which closely approximates the similar curve previously constructed by Webster[4] for this strain; this control curve, since it remains constant throughout, is useful as a graphic indicator of the rise and fall of the other curves in the course of the 12 month period.   This chart may be compared with Text-fig. 1 of the paper already referred to,[1] having been constructed in precisely the same manner.

Comparison of these charts with the corresponding charts of the previous year will show certain differences in the response of the mice to mouse typhoid in the two periods.   The seasonal fluctuation in resistance during the first year (1923–24) followed a fairly regular course—a low mortality rate during the fall and winter, rising to a high peak in the spring (February to May inclusive); a very low level during the summer months of June and July, followed by another peak in September.   In the year just past (1924–25) this same sequence of events is not quite maintained.   The first variation from the standard established in the 1st year is the surprisingly high set

[3] Pritchett, I. W., *J. Exp. Med.*, 1925, xli, 195.
[4] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 231.

Rockefeller Institute Mice
Bagg
Lathrop
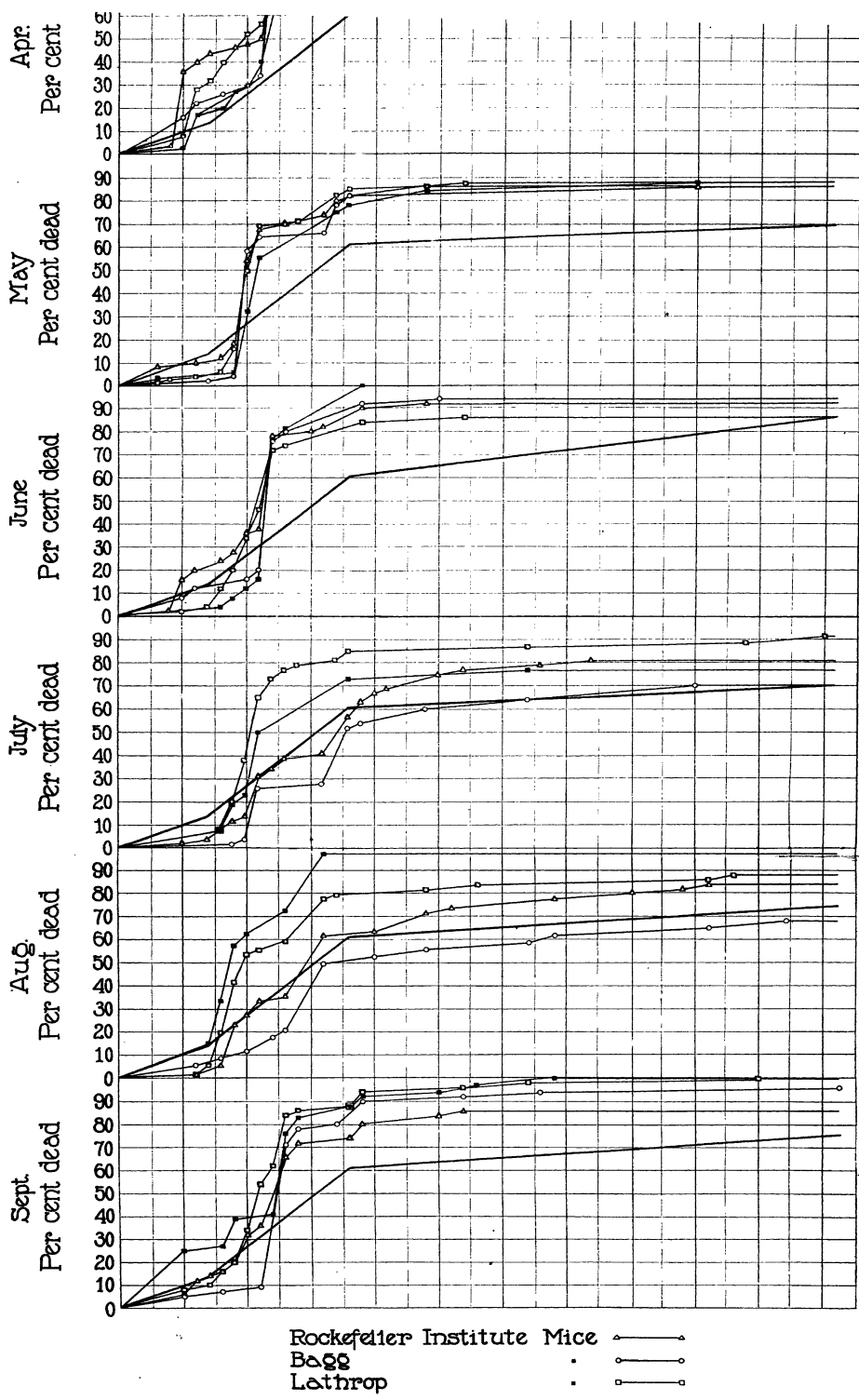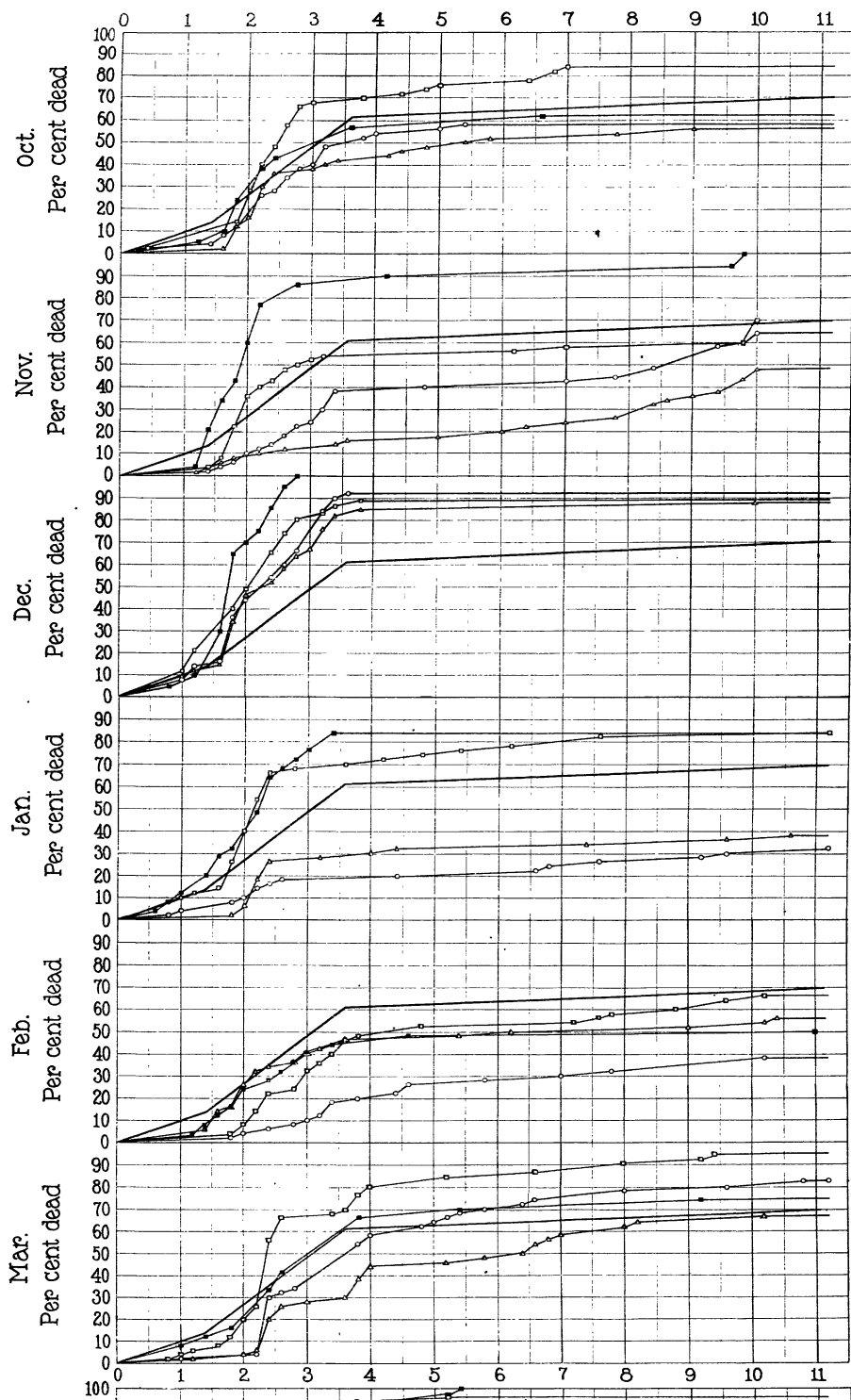
of curves obtained in December, comparable to those seen in the preceding spring months. This peak is followed by two sets of low curves, comparable to those seen in the preceding summer. In March, however, an upward tendency becomes apparent in all strains and the next 3 months—April to June inclusive—show the same high mortality rate that was so striking in the spring months of the year before. As in the preceding year, the July curves were, in general, the lowest for the summer, the Lathrop mice alone showing a lower summer mortality rate in June. Starting with the month of August there is again an upward trend in the curves, except for the Bagg curve, which drops still lower in August. In September all curves are high, their pitch being especially steep.

In Text-fig. 2 the seasonal variation in susceptibility of all strains of mice for the 2 years may be easily compared. In this chart appear the curves constructed last year by plotting for each strain of mice the total mortality occurring in each successive monthly experiment, the mortality figure given in any one month representing the mortality rate for the experiment begun in that month. (These same curves may be found in Text-fig. 2 of the paper already referred to.[1]) Superimposed on these curves are similar curves constructed from the total mortality figures obtained in the monthly experiments of the past year. Average curves for the four strains of mice for the 2 years are also included.

It will be seen that, in general, the same thing has happened in all strains in each of the 12 month periods. There has been a high mortality rate during the spring, a lower summer rate, and a subsequent fall rise. During the winter of 1924–25, however, there seems to have been a secondary rise in the mortality rate of all strains in December, followed by a lower rate in January, and an extraordinarily low rate in February, before the upward trend begins toward the spring peak.

No explanation for the departure from the record of the previous winter can be offered now. Circumstances of an undetermined nature obviously produced a marked simultaneous effect on all the four strains of mice in December, 1924, through which mortality was increased, just as other circumstances, equally unknown, brought about a lowering of the mortality rate during the 2 months following.

Per cent dead

Per cent dead

Per cent dead
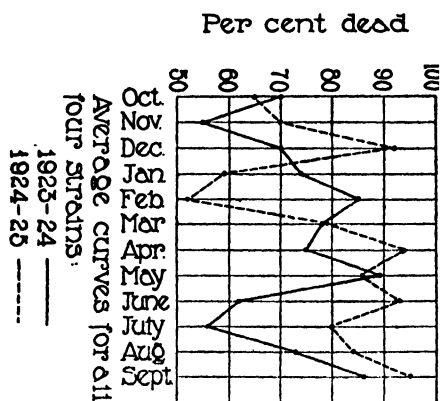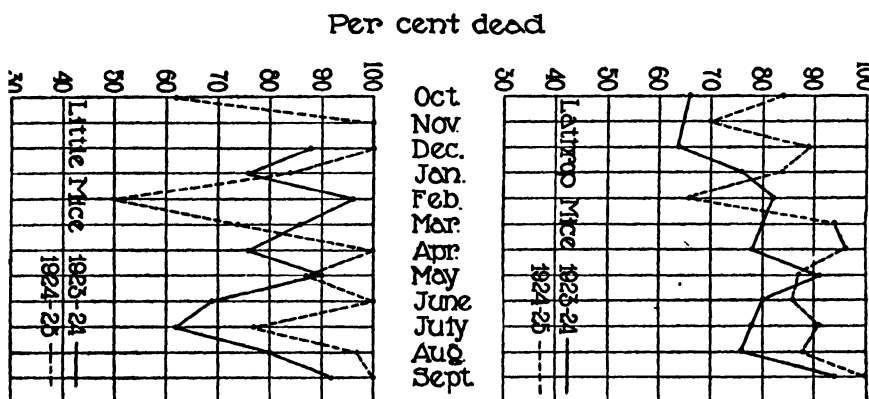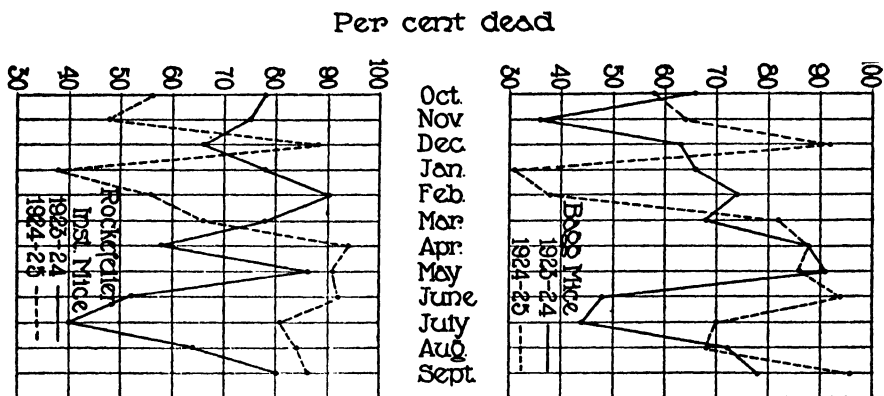
TEXT-FIG. 2.

It is, of course, true that the months of the years are only roughly comparable and variables occurring within them may bring about such effects as we have described. Future studies along the lines of these observations will doubtless lead to clearer perceptions of such influences. In general, however, the findings of the 2 years are in accord and may be taken to establish the fact that a definite seasonal variation in the susceptibility of different strains of mice to a standard dose of mouse typhoid bacilli does occur.

DISCUSSION.

In our previous communication[1] we expressed the belief that "the seasonal variations in mortality rate recorded for these mice are due to fluctuations in host susceptibility." As a basis for this belief we pointed out that the virulence factor of paratyphoid-enteritidis strains is, as Webster[4,5] has shown, quite constant. In our experiments the dosage factor, an important consideration, was kept uniform. The past year's experience has strengthened our view of the fluctuation of host susceptibility. We have systematically employed for the inoculation a stock strain of M. T. II, kept in agar cultures at +4°C., periodically transferred, and submitted to no animal passage for more than 3 years.

It is mere speculation, in view of the paucity of precise knowledge of the subject, to undertake to explain the seasonal fluctuations which we have ascertained and described, just as at present it would not be profitable to discuss the particular variations of the mortality curves observed in December, 1924, and the 2 following months, from the curves obtained in the previous year.

SUMMARY.

Four unrelated strains of mice were tested over a period of 1 year for their seasonal variation in susceptibility to *per os* feeding with a culture of *Bacillus pestis caviæ* of known virulence. Certain consistent fluctuations, determined to be in general agreement with those already recorded in a previous paper, were found and described.

[5] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 781; xxxviii, 33, 45.

EFFECTS OF SPONTANEOUS DISEASE ON ORGAN WEIGHTS OF RABBITS.

By WADE H. BROWN, M.D., LOUISE PEARCE, M.D., AND CHESTER M. VAN ALLEN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In recent papers (1, 2) we reported the results of a study of organ weights in apparently normal rabbits considering, first, a group of 350 animals without reference to any factor that might have affected the results obtained. The second paper dealt with possible effects of obscure lesions on the weights of different organs. It was found that the values obtained differed slightly according to the extent of the lesions present, but in all cases they were within the limits of error for any one of the four groups into which the entire series was divided. It thus appeared that the presence of disease processes produced no marked alteration in the mass or mass relations of organs that were not directly affected by the disease, so long as the animal remained in apparent good health; that is, free from signs and symptoms of disease. It was pointed out, however, that even the small differences noted suggested the occurrence of progressive changes in organ weight which became more and more apparent as the disease progressed to the point of clinical recognition.

A study of organ weights in rabbits presenting obvious manifestations of disease of spontaneous origin was carried out parallel with the study of apparently normal rabbits for the purpose of determining whether any relation could be detected between physical constitution, as represented by organ weight, and functional activity, as measured by the efficiency of the reaction to disease. The object of the present paper is to report the results of this investigation without reference to any effects that might be attributable to the character of the disease or the nature of the lesions. These aspects of the problem will be reserved for future consideration.

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Methods and Material.

The results to be reported in this paper are based on a study of 127 male rabbits comparable in all respects to those used in the study of normal rabbits except for the presence of physical signs or symptoms of disease of spontaneous origin. Most of the animals were originally selected for the normal series or for some other experiment but were not used for such purposes on account of the development of clinical manifestations of disease while under observation; other animals were selected from the general stock to supplement the group obtained in this way.

The diseases presented by animals of this group were, for the most part, chronic affections such as are apt to develop among laboratory stocks. They included chiefly infections of the nasal passages and sinuses (snuffles) together with infections of the middle ear and mastoids, subcutaneous and deep seated abscesses, coccidiosis, nephritis, and bronchopneumonia. An examination was also made of a small group of animals that died or were killed in the terminal stages of such acute diseases as hemorrhagic septicemia, bronchopneumonia, and enterocolitis. The criteria for inclusion of an animal in this series were the presence of such signs or symptoms of disease as a mucopurulent or purulent discharge from the nose, incoordination or ataxia, inflammatory swellings or accumulations of pus in the tissues, persistent loss of weight or weakness and emaciation, and profuse and persistent diarrhea, or death from unsuspected or obscure disease applicable, in particular, to acute infections.

The methods of conducting the investigation were the same as those employed in the case of normal rabbits, including observation for considerable periods of time as a means of making an appropriate clinical classification.

The classification of animals that was used in this study was chosen with reference to the efficiency of the reaction to disease. A primary division of progressive, and non-progressive or regressive conditions was made and each of these was further divided, giving the following groups arranged in the order of decreasing efficiency.

1. Non-progressive affections.
   Effectual reaction.
   A. Chronic disease with definite or well advanced improvement or complete recovery.
   B. Chronic diseases with stationary or uncertain course.
2. Progressive affections.
   Ineffectual reaction.
   C. Chronic progressive diseases.
   D. Acute infections with fatal termination.

This classification assumes that the course of disease is towards death or recovery and that the termination and rate of progress in either direction bear some general relation to the efficiency of the reaction, hence, animals dying acutely would be regarded as displaying the least efficient and those that recover, the most efficient reaction. The grouping of material for analysis and the presentation of results are

based on this general conception. These four clinical divisions form the basis for the grouping of data in Table I under corresponding group designations.

The results are recorded in two forms. The numerical values for organ weights are given in Table I with a normal value for each set of figures to facilitate comparison. In Text-figs. 1 to 7, an effort is made to correlate the results obtained with those obtained for normal rabbits on the basis of mean organ weights and coefficients of variation. For this purpose, two groups of animals are used, one representing the apparently normal and the other the obviously diseased animals. The normal animals are divided into four groups, as in the second paper of this series (2), according to the presence or absence and the extent of the lesions found at autopsy. Group 1 is composed of rabbits that showed no gross lesions, Group 2, of animals with slight lesions, and Groups 3 and 4, of animals with moderate and marked lesions respectively. The diseased animals are subdivided into two classes: those showing relatively high resistance or efficient reactions, and those showing relatively low resistance or ineffectual reactions. The subdivisions of these two groups are explained above.

In this way, we have been able to arrange the values for normal and for diseased rabbits in the form of a progressive series, using postmortem findings in one group of animals as the connecting link with the other. Beginning at the middle of each line and proceeding to either the right or the left, we have, first, the value for the total number of normal rabbits, second, the value for those that showed no lesions at autopsy, etc., with the scale of abnormality increasing or the scale of efficiency diminishing until we reach the groups in which disease was apparent during life. In the case of animals of relatively low resistance (right) the terminal event is death, while in those of higher resistance (left) it is recovery, so that the downward progression in the scale of efficiency is continuous in one case but is ultimately reversed in the other with what may be regarded as a change toward normality.

In the graphic representation of results, mean organ weights are compared on the basis of a percentage deviation from the normal or the value obtained for the 350 normal rabbits, while. in the case of coefficients of variation, the actual values are recorded. In one set of figures (Text-figs. 1 to 4) the organs are grouped and compared first on the basis of mean weights, including both the actual and relative weights, then on the basis of coefficients of variation. In the other figures (Text-figs. 5 to 7), the organs are taken separately in order to bring out the relation of all values for each organ.

The lymph nodes have been omitted from these figures on the basis of insufficient data due to the fact that many of these animals were autopsied prior to the inclusion of lymph nodes in our regular scheme of investigations.

No available data have been excluded in making any of the calculations. In Table I, two values are given for thyroid weights of animals in Group A with a preference indicated for a value obtained by the omission of a weight for one animal which presented a definite goiterous enlargement of the thyroid. The figures given in brackets include this weight. A similar discrimination is to be found in the

TABLE I.

Results Obtained for Body and Organ Weights According to Groups.

Organ.	Group.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	*± gm.*	*± gm.*	*per cent*
Gross body weight.	Normal.	350	2240	2225	1400	3500	353	238	15.7
	A	16	2370	2350	1875	2875	373	252	15.7
	B	53	2385	2275	1325	3475	436	294	18.3
	C	50	2013	2100	1230	2750	345	233	17.1
	D	8	1922	—	1500	2250	236	159	12.3
Gastrointestinal mass.	Normal.	350	416	410	115	710	80.9	54.5	19.4
	A	16	422	410	310	565	74.1	49.9	17.5
	B	53	409	400	290	580	71.1	47.9	17.3
	C	50	376	390	190	595	105.8	71.4	28.1
	D	8	381	—	300	460	49.6	33.4	13.0
Net body weight.	Normal.	350	1849	1800	1150	3010	323	218	17.5
	A	16	1948	1885	1310	2510	313	211	16.0
	B	53	1975	1920	1030	3030	406	274	20.5
	C	50	1637	1675	1015	2305	277	187	16.9
	D	8	1541	—	1153	1850	208	140	13.4
Heart. Actual.	Normal.	348	5.26	5.12	3.2	10.18	0.96	0.64	18.2
	A	16	5.72	5.7	3.74	7.45	0.97	0.65	16.9
	B	53	5.7	5.48	4.0	7.9	1.01	0.678	17.6
	C	50	5.12	5.04	2.7	8.45	1.04	0.7	20.3
	D	6	5.53	—	4.54	7.65	1.05	0.71	18.6

Relative.	Normal.	348	2.85	2.81	1.95	4.42	0.35	0.24	12.3
	A	16	2.95	3.02	2.41	3.35	0.26	0.18	8.8
	B	53	2.92	2.8	2.43	4.48	0.36	0.24	12.3
	C	50	3.15	3.08	2.26	4.95	0.467	0.315	14.8
	D	6	3.84	—	3.19	4.89	0.56	0.38	14.6
Liver. Actual.	Normal.	350	84.3	80.0	50.0	150.0	19.7	13.3	23.4
	A	16	89.2	84.0	60.0	135.0	20.7	13.9	23.2
	B	53	77.4	74.0	43.0	127.0	16.6	11.2	21.4
	C	50	80.8	75.0	30.0	225.0	27.1	18.3	33.5
	D	6	74.8	—	65.0	96.0	11.5	7.8	15.3
Relative.	Normal.	350	46.5	44.9	23.2	97.4	11.84	7.99	25.5
	A	16	46.5	44.6	35.5	84.0	11.67	7.87	25.1
	B	53	40.3	37.3	24.5	78.9	10.4	7.01	25.8
	C	50	49.9	46.9	29.0	157.4	19.01	12.82	38.1
	D	6	51.8	—	37.7	61.9	9.97	6.72	19.2
Kidneys. Actual.	Normal.	348	12.7	12.5	7.15	22.73	2.07	1.4	16.3
	A	16	14.15	13.1	10.04	19.39	3.34	2.25	23.6
	B	53	13.7	13.34	9.63	22.8	2.6	1.75	18.9
	C	50	13.76	12.85	9.17	25.2	3.07	2.07	22.3
	D	6	14.4	—	12.25	21.37	3.16	2.13	21.9
Relative.	Normal.	348	6.97	6.84	3.45	17.28	1.26	0.85	18.1
	A	16	7.22	7.4	5.45	8.53	0.76	0.51	10.5
	B	53	7.02	6.88	4.68	11.65	1.32	0.89	18.8
	C	50	8.68	7.87	5.12	21.17	2.59	1.75	29.8
	D	6	9.9	—	7.37	13.66	2.15	1.45	21.7

TABLE I—*Continued.*

Organ.	Group.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	*± gm.*	*± gm.*	*per cent*
Spleen. Actual.	Normal.	347	0.99	0.9	0.08	3.2	0.436	0.294	44.0
	A	16	1.013	0.82	0.63	2.45	0.448	0.302	44.2
	B	52	1.099	1.05	0.45	2.7	0.512	0.345	46.6
	C	50	1.089	1.02	0.08	3.2	0.714	0.482	65.5
	D	5	0.808	—	0.42	1.82	0.521	0.351	64.4
Relative.	Normal.	347	0.531	0.49	0.035	1.714	0.214	0.144	40.3
	A	16	0.536	0.47	0.298	1.218	0.23	0.155	42.8
	B	52	0.58	0.536	0.205	1.55	0.277	0.187	47.7
	C	50	0.674	0.655	0.05	1.91	0.334	0.225	49.5
	D	5	0.537	—	0.283	1.055	0.278	0.188	51.7
Thymus. Actual.	Normal.	349	2.21	2.13	0.45	5.9	0.848	0.572	38.4
	A	16	1.764	1.6	0.735	3.45	0.643	0.434	36.4
	B	53	2.092	2.09	0.342	3.75	0.73	0.49	34.9
	C	50	1.391	1.16	0.115	3.7	0.947	0.639	68.1
	D	5	1.798	—	0.712	3.28	0.838	0.565	46.6
Relative.	Normal.	349	1.23	1.147	0.334	2.72	0.451	0.304	36.6
	A	16	0.893	0.881	0.44	1.57	0.249	0.168	27.8
	B	53	1.073	1.078	0.203	1.84	0.365	0.246	34.0
	C	50	0.807	0.647	0.069	2.278	0.546	0.368	67.8
	D	5	1.158	—	0.537	2.09	0.503	0.339	43.4

Testicles.								
Actual.								
Normal.	290	4.86	4.85	0.81	9.12	1.63	1.1	35.5
A	16	5.41	4.94	1.32	8 05	1.11	0.75	20.5
B	49	5.2	5.04	2.7	8.56	1.21	0.82	23.2
C	47	4.45	4.58	0.78	9.08	1.73	1.17	38.8
D	6	4.73	—	2.53	6.6	1.42	0.96	30.0
Relative.								
Normal.	290	2.61	2.62	0.47	4.93	0.7	0.47	26.9
A	16	2.74	2.73	1.0	4.03	0.77	0.52	28.1
B	49	2.7	2.59	1.1	4.43	0.6	0.41	22.3
C	47	2.71	2.72	0.5	4.58	0.87	0.59	32.1
D	6	3.17	—	2.19	4.41	0.73	0.49	23.0
Brain.								
Actual.								
Normal.	150	9.31	9.24	7.42	12.03	0.78	0.53	8.4
A	16	9.17	8.9	8.05	11.6	0.87	0.59	9.4
B	45	9.22	9.15	7.05	11.0	0.75	0.51	8.1
C	40	9.25	9.39	7.56	11.84	0.91	0.61	9.7
D	6	9.17	—	8.25	11.39	1.03	0.69	11.2
Relative.								
Normal.	150	5.11	5.06	3.33	8.16	0.87	0.58	16.9
A	16	4.82	4.49	3.6	6.45	0.71	0.48	14.7
B	45	4.75	4.7	3.44	6.28	0.69	0.47	14.5
C	40	5.68	5.43	4.37	9.27	1.01	0.68	17.7
D	6	6.33	—	5.14	7.85	0.97	0.65	15.3

TABLE I—*Continued.*

Organ.	Group.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	*± gm.*	*± gm.*	*per cent*
Thyroid	Normal.	349	0.231	0.185	0.085	1.57	0.162	0.109	70.3
Actual.	A	16	0.252	0.21	0.115	0.55	0.121	0.082	48.0
			(0.37)	(0.214)	(0.115)	(2.14)	(0.472)	(0.318)	(127.5)
	B	53	0.294	0.215	0.13	1.375	0.232	0.157	79.0
	C	50	0.214	0.18	0.115	0.53	0.097	0.066	45.4
	D	8	0.204	—	0.11	0.302	0.062	0.041	30.0
Relative.	Normal.	349	0.123	0.098	0.05	0.73	0.076	0.051	61.5
	A	16	0.131	0.114	0.071	0.315	0.062	0.042	47.2
			(0.176)	(0.117)	(0.071)	(0.851)	(0.179)	(0.121)	(101.4)
	B	53	0.148	0.119	0.063	0.658	0.101	0.068	67.8
	C	50	0.129	0.117	0.067	0.312	0.051	0.034	39.9
	D	8	0.131	—	0.095	0.181	0.031	0.021	23.6
Parathyroids	Normal.	349	0.01256	0.012	0.002	0.035	0.00486	0.00328	38.7
Actual.	A	16	0.0156	0.013	0.007	0.03	0.00623	0.0042	40.0
	B	53	0.0156	0.015	0.005	0.062	0.00814	0.00549	52.2
	C	50	0.0141	0.013	0.004	0.032	0.00584	0.00394	40.0
	D	8	0.0179	—	0.01	0.028	0.0065	0.00438	36.3
Relative.	Normal.	349	0.00692	0.00631	0.00109	0.02175	0.00278	0.00187	40.1
	A	16	0.00824	0.00774	0.00401	0.01363	0.00325	0.00219	39.4
	B	53	0.0078	0.0076	0.00301	0.02046	0.00263	0.00177	33.7
	C	50	0.00876	0.00787	0.00358	0.02415	0.00385	0.0026	43.9
	D	8	0.0128	—	0.0065	0.01695	0.00365	0.00246	28.4
Hypophysis.	Normal.	348	0.028	0.028	0.015	0.044	0.00517	0.00349	18.5
Actual.	A	16	0.0304	0.03	0.022	0.043	0.0051	0.00344	16.7
	B	53	0.0305	0.03	0.02	0.05	0.0066	0.00445	21.6
	C	50	0.02936	0.028	0.015	0.057	0.0073	0.0049	24.8
	D	8	0.0284	—	0.015	0.04	0.0078	0.00526	27.4

Relative.	Normal.	348	0.0154	0.0153	0.00728	0.0294	0.0031	0.00209	20.3
	A	16	0.01588	0.01567	0.01122	0.02095	0.00255	0.00172	16.1
	B	53	0.0158	0.0156	0.0075	0.0279	0.0036	0.00242	22.8
	C	50	0.01812	0.0168	0.0087	0.03304	0.0047	0.0032	25.9
	D	8	0.0185	—	0.0113	0.026	0.0047	0.0032	25.4
Suprarenals. Actual.	Normal.	349	0.3801	0.358	0.115	1.05	0.155	0.1046	40.8
	A	16	0.4514	0.42	0.22	0.79	0.139	0.0938	30.8
	B	53	0.458	0.392	0.15	0.92	0.1927	0.1299	42.1
	C	50	0.4705	0.4	0.157	1.3	0.2442	0.1647	51.8
	D	8	0.6299	—	0.3	1.539	0.3808	0.2568	60.4
Relative.	Normal.	349	0.2088	0.1967	0.0804	0.53	0.075	0.0506	35.9
	A	16	0.2357	0.2156	0.115	0.453	0.0742	0.05	31.4
	B	53	0.231	0.2099	0.0882	0.464	0.0855	0.0577	37.0
	C	50	0.2836	0.262	0.1245	0.613	0.1276	0.0861	45.0
	D	8	0.4008	0.1739	0.1739	0.832	0.2022	0.1363	50.4
Pineal gland. Actual.	Normal.	348	0.01609	0.015	0.005	0.03	0.00429	0.00289	26.7
	A	16	0.0178	0.017	0.012	0.025	0.00309	0.00208	17.3
	B	53	0.0175	0.0155	0.01	0.034	0.0056	0.00378	32.0
	C	49	0.01586	0.015	0.009	0.03	0.00425	0.00287	26.8
	D	7	0.0167	—	0.005	0.04	0.01044	0.00704	62.5
Relative.	Normal.	348	0.00885	0.00868	0.00273	0.01873	0.00245	0.00165	27.7
	A	16	0.00946	0.00974	0.00553	0.01527	0.00223	0.0015	23.5
	B	53	0.00901	0.00829	0.00501	0.0234	0.00308	0.00208	34.2
	C	49	0.00995	0.00962	0.00528	0.01847	0.00293	0.00198	29.4
	D	7	0.01113	—	0.0029	0.02402	0.0061	0.00411	54.8

TABLE I—*Concluded.*

Organ.	Group.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	*± gm.*	*± gm.*	*per cent*
Popliteal lymph nodes. Actual.	Normal.	242	0.2554	0.245	0.085	0.72	0.0824	0.0556	32.3
	A	9	0.2216	0.22	0.13	0.315	0.0624	0.0421	28.1
	B	43	0.2287	0.21	0.115	0.375	0.0656	0.0442	28.7
	C	30	0.2278	0.21	0.075	0.51	0.097	0.0654	42.5
	D	—	—	—	—	—	—	—	—
Relative.	Normal.	242	0.1357	0.1361	0.0525	0.382	0.0487	0.0329	35.9
	A	9	0.111	0.1147	0.0518	0.1719	0.0389	0.0262	35.0
	B	43	0.118	0.11	0.0636	0.2039	0.0344	0.0232	29.1
	C	30	0.141	0.1355	0.0522	0.304	0.0484	0.0327	34.3
	D	—	—	—	—	—	—	—	—
Axillary lymph nodes. Actual.	Normal.	242	0.1759	0.175	0.04	0.4	0.0598	0.0403	34.0
	A	9	0.1516	0.13	0.075	0.23	0.0514	0.0347	33.9
	B	43	0.1482	0.145	0.05	0.41	0.0557	0.0376	37.6
	C	30	0.1284	0.118	0.035	0.275	0.0503	0.0339	39.1
	D	—	—	—	—	—	—	—	—
Relative.	Normal.	242	0.0972	0.091	0.0187	0.242	0.0378	0.0255	38.9
	A	9	0.0753	0.084	0.0299	0.125	0.0279	0.0188	37.1
	B	43	0.0801	0.0751	0.0332	0.282	0.0426	0.0287	51.9
	C	30	0.0786	0.0704	0.0206	0.1652	0.0319	0.0215	40.6
	D	—	—	—	—	—	—	—	—

Deep cervical lymph nodes.								
Actual.	Normal.	124	0.1558	0.14	0.035	0.405	0.0752	0.0494	47.0
	A	4	0.183	—	0.1	0.31	0.0861	0.0544	47.0
	B	24	0.2583	0.22	0.095	0.67	0.1422	0.0959	55.1
	C	15	0.289	0.17	0.115	0.84	0.1921	0.1296	66.4
	D	—	—	—	—	—	—	—	—
Relative.	Normal.	124	0.0821	0.0724	0.0205	0.211	0.0371	0.025	45.2
	A	4	0.0893	—	0.0613	0.1302	0.0256	0.0173	28.6
	B	24	0.1327	0.1113	0.0538	0.325	0.0717	0.0484	54.0
	C	15	0.1774	0.1255	0.0801	0.555	0.1141	0.077	64.3
	D	—	—	—	—	—	—	—	—
Mesenteric lymph nodes.								
Actual.	Normal.	133	3.488	3.32	1.34	9.19	1.31	0.88	37.5
	A	9	3.26	3.27	2.17	4.65	0.68	0.46	20.9
	B	39	2.99	2.9	1.36	4.85	0.827	0.558	27.6
	C	34	2.94	2.9	1.25	5.5	1.01	0.68	34.3
	D	—	—	—	—	—	—	—	—
Relative.	Normal.	133	1.828	1.763	0.72	3.71	0.642	0.43	35.1
	A	9	1.637	1.39	0.912	2.86	0.543	0.366	33.1
	B	39	1.52	1.505	0.714	3.19	0.443	0.299	29.1
	C	34	1.75	1.66	0.821	3.49	0.558	0.376	29.6
	D	—	—	—	—	—	—	—	—

text-figures where the line for the thyroid is continued to the value obtained by omitting this one weight instead of the value with this weight included, as indicated in the figures.

RESULTS.

The results of the observations made on organ weights of rabbits presenting clinical manifestations of disease of spontaneous origin are recorded in Table I and Text-figs. 1 to 7.

DISCUSSION AND CONCLUSIONS.

An examination of the figures given in Table I will show that in most instances the values obtained for the four groups of diseased rabbits do not differ greatly from those of the normal controls.   Mean values for actual organ weight may be larger or smaller and occasionally the maximum and minimum values exceed the normal limits, but weights per kilo of net body weight show a more consistent behavior; mean weights for the heart, liver, kidneys, spleen, thyroid, parathyroids, suprarenals, hypophysis, pineal gland, and deep cervical lymph nodes tend to run high, while those for the thymus, popliteal, axillary, and mesenteric lymph nodes run low; the brain weight is low in Groups A and B and high in Groups C and D.   Similar conditions obtain for the probable error and coefficients of variation.   Still, if the analysis of results were limited to a consideration of the actual magnitude of the deviation from the normal, it would be found that nearly all of the weights are within the limits of error for normal rabbits.

This raises the question of the extent of the deviation from the normal that may be regarded as significant when normal standards are fixed with due regard for circumstances that may affect normal values.   The standards employed in the present instance were worked out with a view to including all variations in weight that are likely to occur in normal rabbits.   They should include, therefore, most functional variations, and from the results recorded in this and the previous paper, together with a large volume of unpublished data, it seems that adequate standards of normality may indeed include all variations in organ weight that are of functional origin and hence all variations that are likely to occur in consequence of disease, except

those of organs that are themselves the seat of some disease process or are directly affected by the disease. Under these circumstances, reliance on differences in weight with corrections for probable error
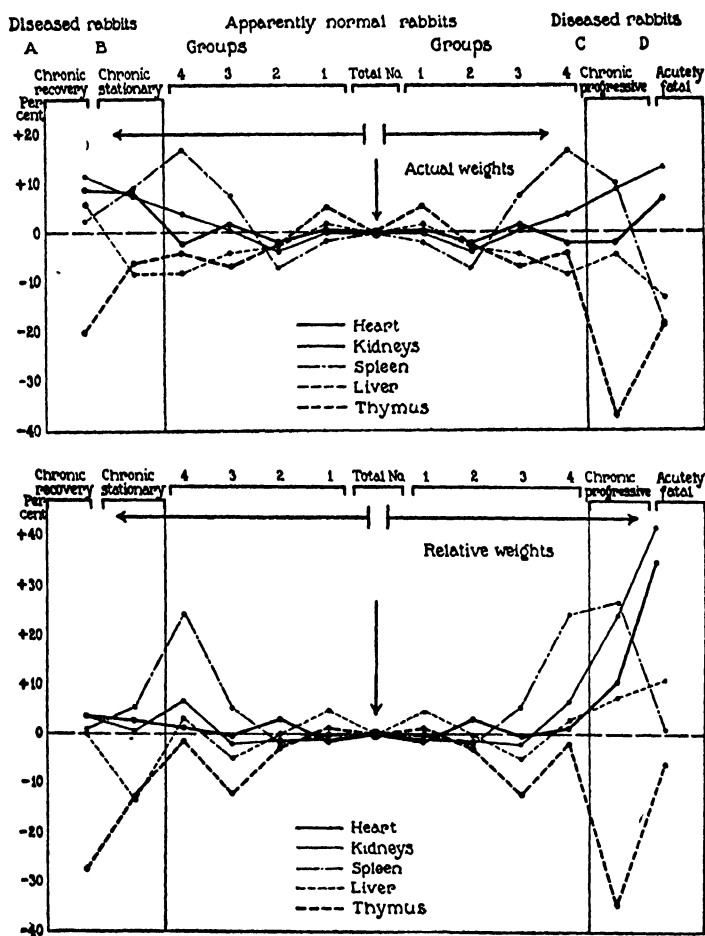
TEXT-FIG. 1. Percentage deviation of actual and relative weights of organs from the mean value obtained for apparently normal rabbits. The values for different groups of animals are arranged according to the extent of lesions found at autopsy or the clinical course of disease.

would tend to disclose only those changes in organ weight that are direct consequences of disease and to obscure those that are due to altered functional activity. Functional alterations are the ones in

TEXT-FIG. 2. Same as Text-fig. 1.
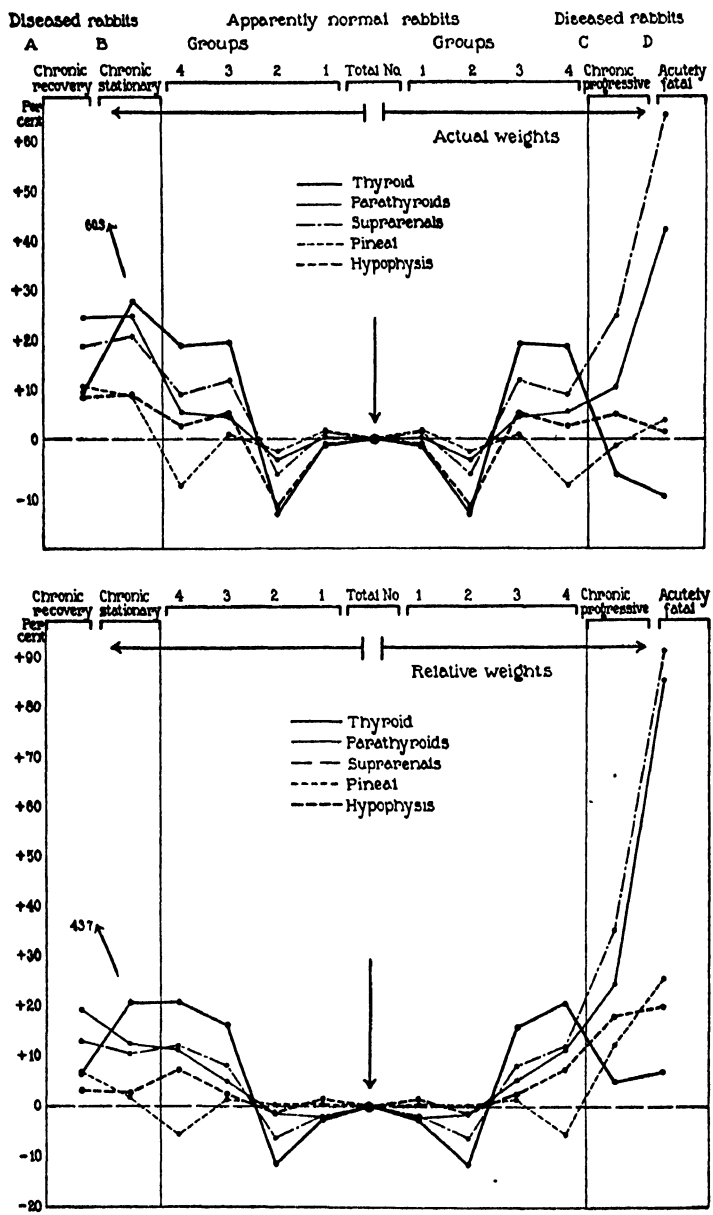
which we are chiefly interested, and the fact that the changes found are within the limits of normal variation is important evidence of their nature.
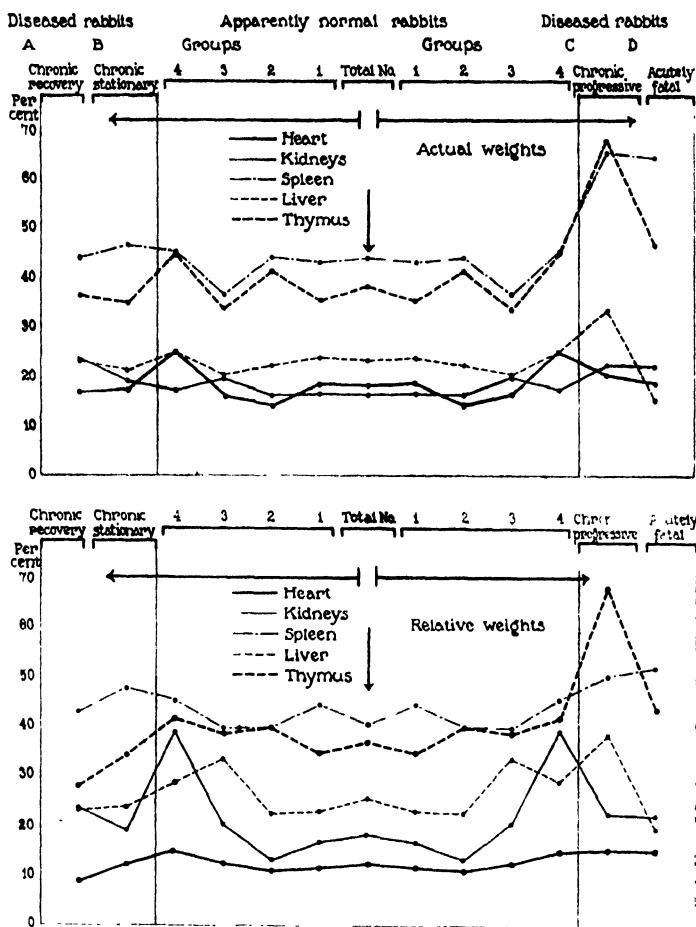
TEXT-FIG. 3. Coefficients of variation for actual and relative weights of organs of apparently normal and diseased rabbits.

If we consider not only the magnitude of the variation but the direction and order of arrangement of values as given, it will be found that there is an indication of a relation between the deviation from the normal and the clinical status of the several groups of animals with respect to the efficiency of the reaction to disease. This is
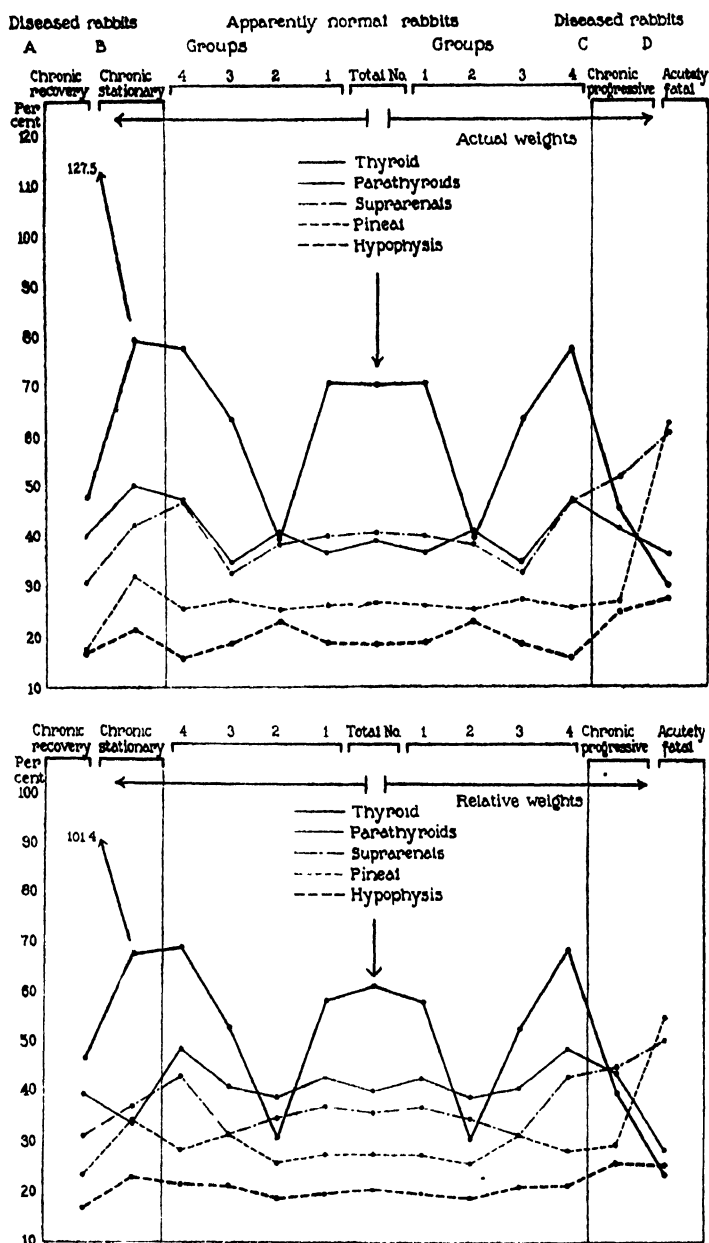
TEXT-FIG. 4. Same as Text-fig. 3.

especially true of Groups A, B, and C which represent animals with chronic affections. As a rule, mean weights per kilo of net body weight for Groups A and B are nearer the normal than those for Group C, and the coefficients are either nearer the normal or smaller. The results for Group D are more irregular; in a few instances they show the closest approximation to the normal or the smallest coefficient of variation but in most cases the relation is reversed. This irregular-
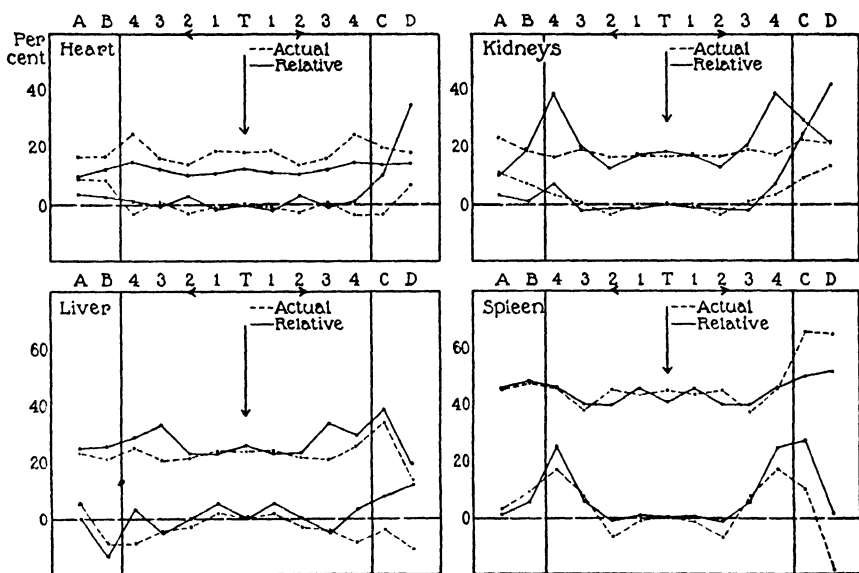
TEXT-FIG. 5. Percentage deviation of weights and coefficients of variation for individual organs as in Text-figs. 1 to 4. The two upper lines represent the values for the coefficients in each case.

ity may be attributable, in part, to the very small number of animals in the group and, in part, to the acute nature of the disease. The group is, however, the most homogeneous of all.

The significance of these findings can be brought out best by correlating the results in animals presenting clinical manifestations of disease with those obtained in apparently normal rabbits, grouped according to the lesions found at autopsy. The results of comparisons of this kind are shown in Text-figs. 1 to 7. In the first series of figures (Text-figs. 1 to 4), organs are grouped and functions are

considered separately, while in the second (Text-figs. 5 to 7), results for individual organs are combined. In analyzing the relationships shown by individual groups of animals, greater significance is to be attached to the form of the curves than to the actual values recorded.

If we consider first the general relationships, as indicated by mean weights (Text-figs. 1 and 2), it will be seen that as we pass from a
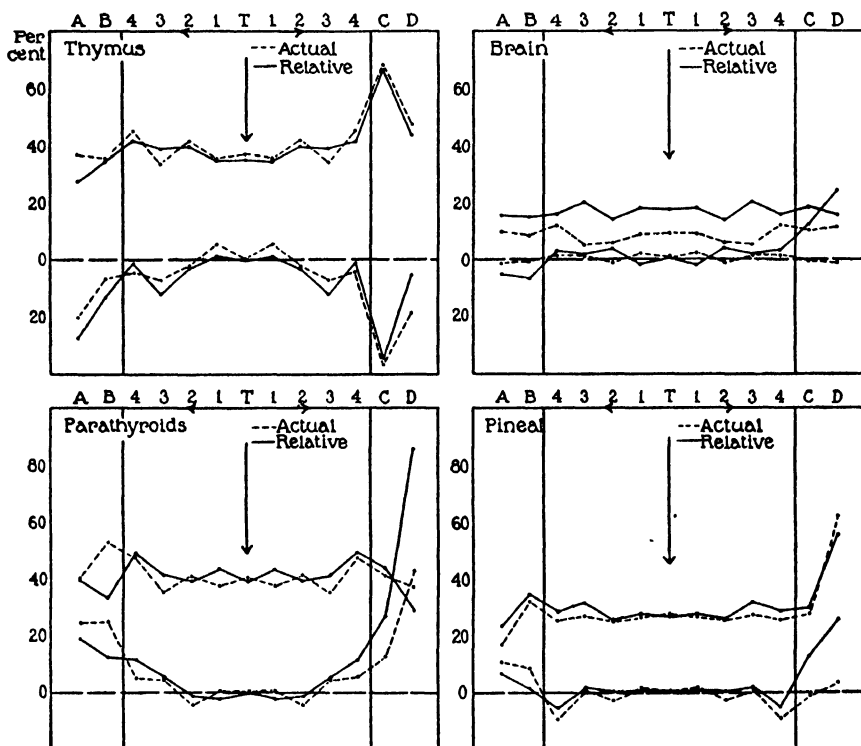
TEXT-FIG. 6. Same as Text-fig. 5.

theoretical normal value through various degrees of abnormality, some organs change very little or not at all while others show decided variations. At first, the lines are closely grouped and they tend to follow a common direction, whether upward or downward, but, as they continue outward in either direction, they diverge or become scattered so that the relation to the normal or of one organ to another differs according to the extent of the lesions present or the clinical

course of disease.   There is, however, a distinct difference between
the conditions shown for animals in which the disease was stationary
or tending toward recovery and those in which the disease was progress-
ing toward a fatal termination.   In the first case, the values change
very little or, in the end, tend to assume a position nearer the normal.
In animals of relatively low resistance, nearly all organs show a con-
tinuation of the lines upward or downward, as the case may be, which
is distinctly greater than that shown on the opposite side of the chart.
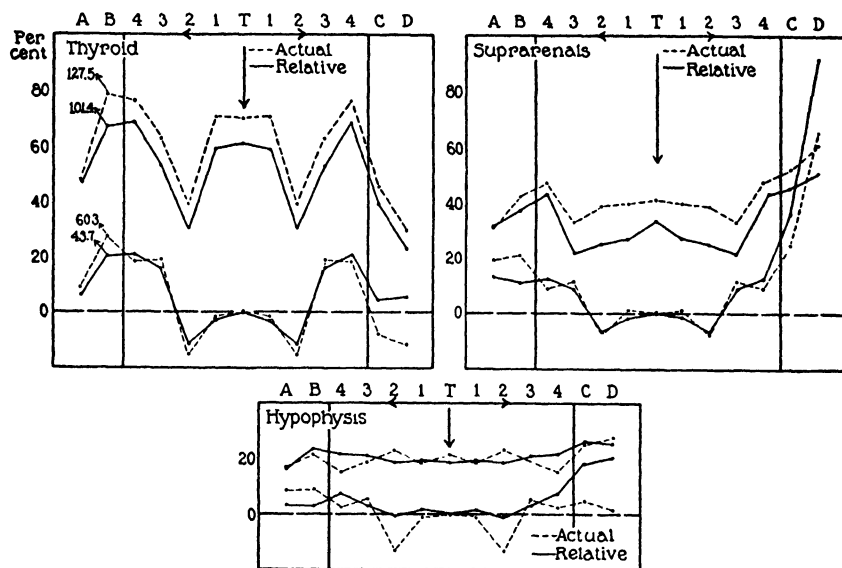
TEXT-FIG. 7.   Same as Text-fig. 5.

Moreover, the general effect is to increase the dispersion of values and
the extent of the divergence from normal.   This is especially applica-
ble to chronic progressive affections and does not apply with equal
force to acutely fatal conditions which may not last sufficiently long
to permit of such changes.

There are striking exceptions to this general rule.   For example,
the thymus shows a reduction in weight for every departure from the
normal and no evidence of a return toward normal with the initiation
of recovery; this doubtless would come as a later development.   Again,
there is a decrease instead of an increase in the weight of the thyroid
in animals with chronic progressive and acutely fatal diseases as

compared with the maintenance of a high weight level in animals that displayed a more effectual resistance. In the light of other evidence, this feature of the thyroid curve appears to be characteristic of the difference between an effectually sustained reaction and an ineffectual or failing reaction.

Coefficients of variation show a similar situation with respect to the constancy of weights in different groups of animals. These values (Text-figs. 3 and 4) are compared on a different basis from mean organ weight with a view to preserving the indication which they afford of the tendency of different organs to vary as well as the tendency of a given organ to vary under different circumstances. This greatly reduces the scale of the variations recorded so that the magnitude of the variation shown cannot be compared directly with that of the change in organ weight. There is, however, a similarity in the form of the two sets of curves, and any decided change in one value is nearly always accompanied by a decided change in the other; these may take the same or opposite directions as shown in Text-figs. 5 to 7.

Without attempting to analyze these changes in detail, it may be said that, in general, the coefficients of variation tend to be low, within the limits of normal, until the borderline groups are reached. At this point, the coefficient is usually increased. In animals that display an effectual reaction (Groups A and B), there is either no further increase or the value diminishes, while in the case of chronic progressive and acutely fatal affections (Groups C and D), the coefficient may remain high, increase, or diminish but, as a rule, the coefficient is larger or the change greater than the corresponding values for Groups A and B; that is, among animals that display an effectual reaction, there is evidence of the preservation of greater constancy with respect to organ weight than among those that display an ineffectual reaction.

The conditions presented by individual organs require no detailed discussion; the form and the magnitude of the change in weight and coefficients of variation as recorded for the several groups of animals are clearly shown in Text-figs. 5 to 7.

This analysis of results shows that the physical constitution of rabbits presenting clinical manifestations of disease differs in several

essential respects from that of normal rabbits. Any departure from the normal appears to be accompanied by some change in organ weight and in the coefficients of variation for organ weight which proceeds parallel with pathological or clinical evidence of disease. The direction of the change varies with different organs but in most instances the first movement is in the direction of a reduction in weight with a smaller coefficient of variation; this change occurs sooner in the glands of internal secretion than in the larger parenchymatous organs (Text-figs. 1 to 4); with increasing abnormality weights and coefficients tend to increase, reaching a maximum in animals that present clinical as well as pathological evidence of disease. At this point the direction of the change varies; if the disease progresses toward a fatal termination most organs show a continued increase in weight while others show an abrupt reduction with irregular changes in the coefficients of variation, but, if the course of events is toward recovery, the organ weights and coefficients tend to diminish or return to normal.

The finding of some difference between the weights of organs of normal and diseased animals is not surprising, as such differences might be caused by a disturbance of nutrition or by retrograde changes in the substance of the organ as direct or indirect consequences of disease. It is not unlikely that these factors, and perhaps others, played some part in the production of the changes found, and if our observations had been limited to animals with outspoken manifestations of disease, it would have been difficult or impossible to determine whether the differences noted represented anything more than results of the action of factors of this kind. With the material available, however, it is apparent that these were not the sole causes.

We have for comparison a group of apparently normal and a group of obviously diseased rabbits. The normal rabbits were in excellent physical condition but many of them (20 per cent) showed lesions at autopsy that were comparable to the majority of those found among the diseased animals. The outstanding difference between the two groups was, therefore, the physical condition or state of nutrition and the presence or absence of other clinical signs and symptoms of disease. Among the diseased animals there were also differences. Most of them were well nourished and, while the proportion of thin or emaciated animals in Group C was larger than that of the other groups,

all groups contained a few such animals; among the animals with chronic diseases, the lesions were also comparable, so that the chief difference was again not one of nutrition or of extent of lesions but the capacity for the control of the disease as indicated by the progress toward death or recovery.

When the results for normal and diseased animals are arranged in serial order, and analyzed with reference to the conditions presented by successive groups, it is found that the changes in organ weight and coefficients of variation do not conform to any scheme of nutritional gradations or even to the extent of lesions; they tend, however, to be consecutive and to follow the course of disease, showing a departure from the normal which, in general, is inversely proportional to the efficiency of the reaction displayed by the several groups of animals. Moreover, the changes that occur are in most cases within the limits of functional variations for normal rabbits.

It appears to be reasonably certain, therefore, that the changes in organ weight and coefficients of variation are of the order of functional reactions which are modified to some extent by other factors.

SUMMARY.

A group of 127 rabbits presenting clinical manifestations of disease of spontaneous origin was studied with a view to determining whether any relation could be detected between physical constitution as represented by organ weights and functional activity as measured by the efficiency of the reaction to disease. The results of the investigation are presented in tabular form and the values obtained are compared, by means of graphs, with corresponding values for normal rabbits.

It was found that animals that were the subjects of disease showed decided changes in the weights of nearly all organs, changes which appeared to be of functional origin in that the values obtained for organ weights and coefficients of variation tended to conform, in general, with the efficiency of the reaction displayed by the various groups of animals into which the entire series was divided.

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EFFECT OF THYROIDECTOMY AND OF THYMECTOMY IN EXPERIMENTAL SYPHILIS OF THE RABBIT.

By LOUISE PEARCE, M.D., and CHESTER M. VAN ALLEN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In previous papers we have emphasized the importance of the integrity and balance of the system of the glands of internal secretion as one of the factors participating in the reaction of the host to the growth and fate of a transplantable neoplasm of the rabbit (1). This conception was primarily based upon well defined gross and histologic changes in a number of the endocrine glands, and it was felt that there is an essential connection between the growth of the transplanted tumor and certain members of the endocrine system, on the one hand, and the mechanism of animal defense, on the other. Both the thyroid and the thymus glands, in particular, appeared to be profoundly affected, and it was found (2) that the malignancy of the neoplasm was greatly increased in those rabbits in which the thyroid gland was completely removed either before or shortly after inoculation, while a partial thyroidectomy usually resulted in a disease of lessened malignancy. The effect of thymectomy was less clear.

In rabbits infected with *Treponema pallidum*, changes in the glands of internal secretion were also found and these in turn could be related to the mechanism of animal defense as expressed by the clinical manifestations of the disease (3). In order to test further the conception of the importance of this system of organs in the reaction of the host to disease, experiments were undertaken in which the thyroid or thymus glands of rabbits were removed prior to inoculation with *Tr. pallidum*. The results are here reported.

Methods and Material.

Two experiments are reported. The series of animals of the first experiment comprised seven rabbits with a complete thyroidectomy, six with a partial thyroidectomy, and six controls. They were inoculated on January 26, 1923. The opera-

tions were performed 7 and 10 days before inoculation in the case of complete removal of the gland, and 9 days in the case of partial removal. A second series were inoculated on October 29, 1923, and comprised five rabbits with a complete thyroidectomy performed 12 and 13 days previously, five rabbits with partial thyroidectomy performed 14 days previously, six rabbits with complete thymectomy performed 19, 21, and 24 days previously, and eight controls.

All operations were carried out under ether anesthesia. In the case of partial thyroidectomy, one lobe and the isthmus of the gland were removed; while the thymus was totally removed by a transthoracic operation (4, 5). At the end of the experiments, 4 months after inoculation of the first series and 7 months after that of the second, all animals were killed with ether and a complete autopsy performed. A search for thyroid or thymus fragments was made at this time. In two instances a tiny nodule of thyroid tissue was found.

The rabbits were young adult or adult males, matched according to age and breed from a selected stock. They were separately caged and fed a standard diet of hay, oats, and green vegetables.

The strain of *Tr. pallidum* used was the Nichols strain, isolated in 1912, and carried in this laboratory since 1917. Inoculations were made in one testicle with 0.2 cc. of a salt solution emulsion rich in treponemata as determined by dark-field examination. The tissue used for the emulsion was derived from an actively progressing orchitis.

The effect of the various operative procedures was studied in relation to the clinical course of the disease. Each rabbit was examined at frequent intervals and notes made of the general physical condition and disease manifestations. Particular attention was paid to the incubation period, the type and duration of the primary orchitis, the involvement of the uninoculated testicle and of the epididymides and scrota, and the occurrence and course of lesions in remote parts of the body such as the bones and periosteum, the skin and mucous membranes, and the eyes. The duration of the experiments, 4 and 7 months, was determined by the fact that, in the majority of instances after testicular inoculation, healing of syphilitic lesions other than minor residual lesions of the genitalia occurs within 4 months, although certain rabbits may continue to show persistent or recurrent lesions, particularly of the eyes and skin, for longer periods.

The results of the experiments are summarized in a series of tables of which Tables I and III contain abbreviated clinical records of individual animals. A general classification of the grades of infection is given in Table II, with the terms slight, fair, moderate, well marked, and severe. In Tables IV and V we have attempted to indicate graphically the character of the infection of each animal as determined by the lesions developed. Four sizes of solid circles are used. The smallest circle indicates a slight lesion or one of short duration, and in the case of extensions and secondary manifestations, few or transient lesions. The next larger circle indicates a more pronounced lesion or a slight one of long duration or more numerous, more marked, or more lasting extensions and secondaries. The more severe lesions, either of grade, number, or persistence, are designated by the

third and fourth size circles.   For convenience, scrotal involvement has been grouped with lesions of the epididymides under the heading of extensions.   In the last column of Tables IV and V, the grade of infection of each animal, as shown by the manifestations of the disease, has been designated by a series of one to five plus signs signifying a slight to severe condition.

<div align="center">RESULTS.</div>

The results of the experiments are tabulated in detail so that only the essential features will be referred to.

Considering first the control animals, it is evident that the disease in the first series, Table I, was definitely more severe than in the second, Table III.   Such differences in experimental syphilis of the rabbit are not infrequently encountered and, as will be seen later, can definitely influence the results of a particular experiment.   Furthermore, well marked individual variations in the disease practically always occur in any single group of five to ten animals.   The disease of the control rabbits of the first experiment may be described as of average grade taking into consideration both the genital[1] and the generalized lesions (Tables I and IV).   Generalized manifestations occurred in five of the six rabbits (84 per cent) and numbered 25 definite lesions, 5 of the bones and periosteum, 18 of the skin, and 2 of the eyes.

The experiment was ended 4 months after inoculation, at which time active but non-progressive syphilitic lesions of the genitalia were still present in four rabbits and, in addition, the subsiding keratitis in two of these animals could still be seen (Table I).   Two of the group had been free from any clinical manifestations for 9 and 14 days respectively.   In this control group of six rabbits the infection taken as a whole may be termed moderate to well marked in three, fair in one, and slight in two animals respectively (Table II).

The disease which developed in the animals in which a complete or partial thyroidectomy (Groups A and B, Table I) had been

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[1] We have not attempted to separate what might properly be considered true metastatic lesions of the scrota from those which develop as extensions from lesions of the testicle or tunics or from those occurring at the site of needle puncture at the time of testicular inoculation.   Some involvement of the epididymis probably always occurs as a direct extension from the testicular process, and frequently the lesions persist after regression and healing of the orchitis.   We have included only those instances which were clinically unmistakable.

TABLE I.

Course of Infection. Experiment 1.

Group and No. of rabbit	Incubation period — Orchitis Primary (days)	Orchitis Metastatic (days)	Scrotal lesions Right (days)	Scrotal lesions Left (days)	Bones and periosteum (days)	(No.)	Skin (days)	(No.)	Mucous membrane (days)	(No.)	Eyes (days)	(No.)	Total No.	Freedom from lesions (days)	Time after inoculation (days)	Residual syphilitic lesions (Postmortem examination)
A 1	10	39									98	(2)	2	0	117	Left orchitis, periorchitis, and epididymitis. Right and left keratitis.
2	14	35	35				73	(1)					1	15	123	Negative.
3	10	35		73	45–66	(8)	45–112	(12)	73–98	(2)	73	(2)	24	0	125	Left orchitis. Right and left keratitis. Lesions of bones and skin.
4	10	35	35		47–52	(6)	47–68	(28)	68	(1)	73	(2)	37	0	116	Right scrotal lesion.
5	10	32	34		45–61	(4)	55	(2)	73	(1)	81	(2)	8	10	122	Negative.
6	10	35	52	68									1	0	123	Right periorchitis. Right scrotal lesion. Left orchitis, periorchitis, and scrotal lesion.
7	10	44											0	24	118	Negative.
	10.5 (av.)	36.4 (av.)			18		43		4		8		73	16.3 (av.)		

B												
1	24	55	46						0	20	118	Negative.
2	21	52	35						1	20	118	"
3	21	44	61					61 (1)	0	0	123	Right epididymitis?
4	29	59	39						0	25	123	Negative.
5	21	59		61 (1)		55 (1)			2	8	119	"
6	21	61						103 (1)	1	35	116	"
	22.8 (av.)	55.0 (av.)		1		2		1	4	21.6 (av.)		
C												
1	10	48	52						0	9	117	Negative.
2	24	46	61				115 (1)		1	0	123	Right epididymitis. Right keratitis.
3	10	46	52	83	55–68 (2)				2	0	123	Right orchitis, periorchitis, and epididymitis. Right scrotal lesion.
4	14	44	55	89	61 (1)	55–89 (9)		119 (1)	11	0	122	Right orchitis, periorchitis, and epididymitis. Left orchitis. Left keratitis.
5	10	44	32	44	53–68 (2)	55–68 (4)			4	14	119	Negative.
6	7	41	52			55–80 (5)			7	0	116	Right and left epididymitis.
	12.5 (av.)	44.8 (av.)		5		18		2	25	11.5 (av.)		

Group A. Complete thyroidectomy.
" B. Partial "
" C. Controls.

TABLE II.

Classification of Grade of Infection.

	Complete thyroidectomy.	Partial thyroidectomy	Complete thymectomy.	Controls.
Experiment 1.				
Severe, +++++	2 (Nos. 3, 4)			1 (No. 4)
Well marked, ++++				2 (Nos. 5, 6)
Moderate, +++	1 (No. 5)	1 (No. 5)		1 (No. 3)
Fair, ++	3 (Nos. 1, 2, 6)	5 (Nos. 1, 2, 3, 4, 6)		2 (Nos. 1, 2)
Slight, +	1 (No. 7)			
Experiment 2.				
Severe, +++++	1 (No. 1)			1 (No. 8)
Well marked, ++++	2 (Nos. 4, 5)	1 (No. 1)	2 (Nos. 1, 4)	2 (Nos. 4, 7)
Moderate, +++	2 (Nos. 2, 3)	4 (Nos. 2, 3, 4, 5)	4 (Nos. 2, 3, 5, 6)	5 (Nos. 1, 2, 3, 5, 6)
Fair, ++	1 (No. 6)			
Slight, +				

performed shortly before inoculation differed in many respects from that of the controls. Considering first the complete thyroidectomy group, the disease as a whole was much more marked. The average incubation period of the primary orchitis was 2 days shorter than in the controls, while the metastatic orchitis developed 8 days earlier and the general character of the genital lesions was generally more severe than in the controls.

A most striking difference in the completely thyroidectomized rabbits was in the generalized lesions (Group A, Tables I and IV).

There were 73 secondary lesions among six of the seven animals of the group; that is, twice as many per animal as in the controls. They were distributed as follows: bones and periosteum 18, skin 43, mucous membranes 4, eyes 8. Their time of appearance was generally earlier than in the controls, and, in addition, their duration was longer, as shown by the presence of a number of lesions 4 months after inoculation (Table I, last column).

The general character of lesions in the completely thyroidectomized rabbits was rather peculiar. The primary and metastatic orchitis and many cutaneous granulomata, including those of the scrota, were extensive processes with an unusual degree of boggy induration which gradually became extremely hard. Regression and resolution of all lesions proceeded slowly, and, in the case of the skin granulomata, there was a noticeable tendency toward the persistence of dry, indolent, skin nodules or patches of thickening. Renewed activity of all types of lesions with a return of progressive growth is not infrequently observed in normal rabbits but this characteristic was more noticeable in the thyroidectomized animals and contributed to the longer duration of the clinical manifestations of the disease.

At the end of the experiment, four thyroidectomized rabbits still showed active syphilitic lesions while the residual lesions in the control animals were less marked and were, for the most part, regressing. The relative proportion of the various grades of infection, as seen in Table II, was about the same as in the controls, but in two operated rabbits, Nos. 3 and 4, the disease was very severe. No other rabbit in the entire experiment approached these in the number, variety, and severity of generalized lesions.

The group of partially thyroidectomized rabbits (Table I, Group B) showed a disease picture quite different from that of either the controls or completely thyroidectomized animals in that the manifestations of the infection were of an extremely mild or benign type. The incubation period of both the primary and metastatic orchitis

TABLE III.

Course of Infection. Experiment 2.

Group and No. of rabbit.	Incubation period. Orchitis. Primary. (days)	Incubation period. Orchitis. Metastatic. (days)	Scrotal lesions. Right. (days)	Scrotal lesions. Left. (days)	Bones and periosteum. (days)	Bones and periosteum. (No.)	Skin. (days)	Skin. (No.)	Mucous membrane. (days)	Mucous membrane. (No.)	Eyes. (days)	Eyes. (No.)	Total No.	Freedom from lesions. (days)	Time after inoculation. (days)	Residual syphilitic lesions.
A																
1	9	39			54–73	(8)			149	(1)	73	(2)	11	29	220	Negative.
2	14	35	44		59–63	(4)			154	(1)	73–86	(2)	7	24	220	"
3	14	38	42		65	(1)	154	(1)	141–154	(2)			4	0	220	Penis. Anus.
4	14	39			59	(1)			167	(1)	80–170	(2)	4	0	220	Right periorchitis and epididymitis. Left periorchitis. Anus. Necrosis of nasal bone.
5	14	38	32	127	54–59	(3)					88	(2)	5	0	220	Right and left periorchitis.
6	9	32	44										0	0	220	Left epididymitis.
	12.3 (av.)	36.8 (av.)			17		1		5		8		31	26.4 (av.)		
B																
1	21	54	28	77	73	(1)	89–127	(6)					7	30	221	Negative.
2	18	50	73										0	115	221	"
3	18												0	122	221	"

No.		(av.)						(av.)		
4	21	51	59	1	54 (1)	66–77 (2)	0	97	221	Negative.
5	18				(1)		3	115	221	"
(av.)	19.2	51.6			7		10	95.8		
C										
1	18	47		63–73 (4)	63–98 (3)	89–127 (1)	4	47	224	Negative.
2	9	?		63 (1)			0	87	224	"
3	18	47	73				1	70	224	"
4	18	42					4	70	224	"
5	18	59					0	119	224	"
6	21	54					0	126	224	"
(av.)	17.0	49.8		5	3	1	9	86.5		
D										
1	18	42	73	59 (1)	59 (1)	149 (1)	1	23	219	Negative.
2	14	42	59	59–63 (2)	59 (1)		0	114	219	"
3	14	35	32	59–73 (2)		175 (1)	0	96	219	"
4	14	38	44		98 (5)	119 (1)	2	96	219	"
5	14	47	77			102 (1)	1	121	219	"
6	32	50					3	37	219	"
7	14	?					8	97	220	"
8	14	42						83	220	"
(av.)	16.7	42.2		5	7	4	16	83.3		

Group A. Complete thyroidectomy.
" B. Partial "
" C. Thymectomy.
" D. Controls.

was significantly longer (Table I, Group B), and the genital lesions, as a whole, were less marked and of shorter duration than those in the other two groups (Table IV).

The mild character of the infection in these partially thyroidectomized rabbits is particularly well illustrated by the extremely small number of generalized lesions—only four—which occurred in three of the six animals (Tables I and IV). In one rabbit there was a small skin granuloma, and in another, one bone lesion; while in the third animal there was a skin granuloma and a unilateral keratitis. These lesions developed at about the same time as those of the controls but were of relatively short duration and all were healed before the end of the experiment.

There was one rabbit in the partial thyroidectomy group, No. 5, with an infection which has been classified as of fair grade, comparable to that of No. 3 in the controls or of Nos. 1, 2, and 6 in the completely thyroidectomized group, while the disease of the other five rabbits was only of a slight grade (Table II).

In the second experiment the differences in the disease picture of the various groups are somewhat less striking, perhaps because the infection itself was on a lower plane of severity.

This is shown by the longer incubation period of the primary orchitis (Table III), the less pronounced character of this and other genital lesions, and the fewer number of generalized manifestations (Table V). There were 16 secondary lesions distributed as follows: bones and periosteum 5, skin 7, eyes 4. The bone lesions and one of the skin occurred in the usual time, that is 2 months after inoculation, but five skin granulomata developed much later than usual (Rabbit 8), as was also the case with three of the four instances of a keratitis.

This experiment lasted 7 months and at its termination no residual syphilitic lesions in the control rabbits were found clinically or in the gross at postmortem examination. The average time in which there had been no clinical manifestations of the disease was 83 days. In classifying the types of infection shown by these rabbits, one animal, as in the first experiment, No. 8, is graded as having a disease of moderate severity, in two, Nos. 4 and 7, it was fair, while in the remaining five it was of slight degree (Table II).

There were six rabbits from which the thyroid gland had been removed before inoculation. As in the case of Experiment 1, the disease

was again more severe in them than in the controls.   The incubation period of the primary and metastatic orchitis was shorter than in the controls, and the genital lesions, as a whole, were more severe (Tables III and V).

TABLE IV.

Character of Infection as Determined by Lesions Developed.

Experiment 1.

Group and No. of rabbit	Genital lesions				Generalized lesions			Grade of infection
	Primary orchitis	Metastatic orchitis and peri-orchitis	Extensions		Skin and mucous membrane	Bones and periosteum	Eyes	
			Scrota	Epid-idymides				
A 1	●	●		●			●	++
2	●	●		●	●			++
3	●	●	●	●	●	●	●	++++
4	●	●	●	●	●	●	●	++++
5	●	●	●	●	●	●	●	+++*
6	●	●		●	●			++
7	●	●		●				+••
B 1	●	●	●	●				+
2	●	●	●	●				+
3	●	●	●	●				+
4	●	●		●				+
5	●	●	●	●	●		●	++
6	●	●		●		●		+
C 1	●	●		●				+
2	●	●•••	●	?			●	+
3	●	●	●	●		●		++
4	●	●	●	●	●	●	●	+++
5	●	●	●	●	●		●	+++
6	●	●	●	●	●	●		+++

Group A Complete thyroidectomy
     • B Partial   "
     • C Controls

     • Residual, hyperplastic thyroid nodule
    •• Extensive mange and wound abscess
   ••• Left testicle castrated 88th day

Generalized lesions developed in five of the six thyroidectomized rabbits (Table III).

There were 17 lesions of the bones and periosteum, 1 of the skin, 5 of the mucous membranes and mucocutaneous borders, and 8 of the eyes, a total of 31 as contrasted with 16 generalized lesions in six of eight controls.   The lesions of the bones and periosteum developed at about the same time as those in the normal

animals, but the majority of eye lesions occurred much earlier; that is, within 73 to 88 days after inoculation. There were two lesions of the mucous membrane of the penis and three of the anus developing about 5 months after inoculation, and

TABLE V.

Character of Infection as Determined by Lesions Developed.

Experiment 2.

Group and No. of rabbit	Genital lesions				Generalized lesions			Grade of infection
	Primary orchitis	Metastatic orchitis	Extensions		Skin and mucous membrane	Bones and periosteum	Eyes	
			Scrota	Epididymides				
A 1	●	●	•	●	•	●	●	++++
2	●	●	•	•	•	•	•	++ *
3	●	•		●	•	•		++
4	●	•	●	●	•	•	●	+++
5	●	•	●	●		•	●	+++
6	●	•	•	●				+ **
B 1	•	•	●	•	●	•		++
2	•	•	•	•				+
3	•			•				+
4	●		●					+
5	•	•			•		●	+
C 1	●	•		•		●		++
2	●	?		•				+
3	•	•		•			•	+
4	●	•		●	•	•		++
5	●	•	•	●				+
6	●	•		•				+
D 1	●	●	•	•			•	+
2	●	•		•				+
3	•	•		•				+
4	•	•	●	•	•	•		++
5	●	•	●	•				+ ***
6	•	•	•	•			•	+
7	●	?		•		•	•	++
8	●	•	•	•	•	•	•	+++

Group A Complete thyroidectomy
"   B Partial thyroidectomy
"   C Thymectomy
"   D Controls

*Residual, pinhead thyroid nodule
**Multiple cutaneous and pulmonary abscesses
***Multiple pulmonary abscesses

again, as in the thyroidectomized rabbits of the first experiment, these were the only lesions of this class in the entire series. The one instance of cutaneous involvement developed in association with a lesion of the adjacent mucous membrane of the anus.

At the end of the experiment four rabbits showed residual syphilitic lesions, while none were found in any of the other animals of the series (Table III).   In Rabbit 3 the regressing lesions of the penis and the anus were still present; the left epididymis of No. 6 showed a slight granulomatous enlargement; in Rabbit 4 there was a residual right and left periorchitis, a right epididymitis, some thickening of the anal mucosa, and a residual necrosis of the nasal bone; in Rabbit 5 there was an inactive bilateral periorchitis.

We have classified the infection of the thyroidectomized rabbits in this experiment as follows: well marked, 1; moderate, 2; fair, 2; and slight, 1, as shown in Table II.

The disease of the five partially thyroidectomized rabbits was, as in the first experiment, of a more benign character than that of the controls.

The primary orchitis was not as severe and the average incubation period was 3 days longer (Tables III and V).   Furthermore, the orchitis of all the partially thyroidectomized rabbits was not clinically apparent before 18 to 21 days, while it was definitely so in six of the eight controls within 14 days.   The metastatic orchitis in seven of the eight controls developed after an average incubation period of 6 weeks, but among the five partially thyroidectomized rabbits a similar lesion had occurred in only three after 51 days.

Generalized lesions were found in only two of the five rabbits comprising this group, an animal incidence of 40 per cent as compared with an incidence of 75 per cent in the controls (Table III).

There were 10 such lesions, 1 of the bones and periosteum, 7 of the skin, and 2 of the eyes, and 7 of them occurred in one rabbit, No. 1, obviously the least resistant animal of the group. Rabbit 5 had one cutaneous granuloma and a bilateral keratitis which developed in the unusually short time of 2 months after inoculation.   .

The duration of both the genital and secondary lesions in this group was relatively short, as is shown by the length of time preceding the close of the experiment in which no lesions were found; that is, 30 to 122 days, or an average of 96 days.   In the case of the controls this period was 83 days, while it was but 27 days for the only two completely thyroidectomized rabbits which were negative at the conclusion of the experiment.

In classifying the grades of infection of individual animals in the group, as shown in Table II, we have considered the disease of Rabbit 1 as representing a fair infection and that of the remaining four as slight.

The last division in the experiment consists of a group of six rabbits in which the thymus gland had been completely removed prior to inoculation (Table III, Group C). The infection which developed in these animals was, in general, like that of the partially thyroidectomized rabbits, less severe than in the controls, yet with certain differences.

The primary orchitis was of the same order as that of the controls, but the metastatic orchitis developed a week later and was slightly less pronounced (Tables III and V). By referring to Tables III and V it will be seen that the primary orchitis and the epididymitis of the thymectomized and control rabbits were, on the whole, quite similar, but that as regards the metastatic orchitis and scrotal involvement the thymectomized rabbits more nearly resembled the partially thyroidectomized animals.

As concerns generalized lesions, the thymectomized rabbits were also more like the partially thyroidectomized animals.

There was almost the same total number of lesions, that is nine in one and ten in the other; these occurred in two of five partially thyroidectomized and in three of six thymectomized rabbits. The lesions were distributed as follows: bones and periosteum 5, skin 3, eyes 1 (Tables III and V). Their time of appearance, general character, and duration presented no unusual features.

At the conclusion of the experiment all thymectomized animals had been clinically free from syphilitic manifestations for 47 to 126 days, or an average of 87 days. In this respect the group resembles the controls with an average figure of 83 days rather than the partially thyroidectomized animals whose average time was 96 days.

The degree of infection of the individual rabbits of the group has been classified as follows: fair, 2; slight, 4; as shown in Table II. This grouping resembles that of the partially thyroidectomized rabbits.

DISCUSSION.

The experiments reported indicate that, in rabbits, surgical removal of the whole or a part of the thyroid gland or of the entire thymus gland

shortly before inoculation with *Tr. pallidum* is followed by well defined differences in the clinical manifestations of the disease.   In the case of complete thyroidectomy, the effect was, in general, one of increased severity as manifested especially by the shortened incubation period and pronounced grade of both the primary and metastatic orchitis, the much higher incidence of generalized lesions, and the distinct tendency for all lesions to be more enduring than in the control animals or, it may be added, than is ordinarily the case in normal rabbits.   Partial thyroidectomy, on the other hand, resulted in a disease that was generally less severe than that of the controls as shown by the milder character of the primary and metastatic orchitis but especially by the low incidence of generalized manifestations and by the relatively short duration of all the lesions.   These contrasting effects of complete and partial thyroidectomy occurred in both experiments, although, as has been pointed out, they were more marked in one than in the other.

It is not possible to speak so definitely about the effect induced by ablation of the thymus since it was studied in but one experiment and, as it happened, the less favorable one for a demonstration.   The disease which developed in the group of rabbits with a complete thymectomy was of a mild type, much less severe than in the group of completely thyroidectomized animals and, on the whole, somewhat less so than that of the controls.   This was particularly evident in the metastatic lesions of the genitalia and the number, incidence, and distribution of generalized manifestations.   In many respects the general plane or grade of infection was similar to that of the group of partially thyroidectomized rabbits.

Because of the variations in the character of syphilitic lesions shown by individual animals in any series of five to ten rabbits, we have discussed the effects induced by various surgical procedures on a group basis.   However, the disease manifestations in certain rabbits were of particular interest.

In the first set of completely thyroidectomized rabbits there were two animals (Table I, Group A, Rabbits 3 and 4) and in the second set there was one (Table II, Group A, Rabbit 1) in which the disease was considerably more severe than in any other animal of either series.

In the second experiment, there was one rabbit (Table III, Group B, Rabbit 1)

in which partial thyroidectomy was followed by more marked disease manifestations than in the other rabbits of this group. A somewhat similar instance is that of Rabbit 4 in the thymectomy group (Table III, Group C). In both animals there was but one bone lesion, a fact which would indicate that at this time the resistance of the host was on a comparatively high level. Subsequently, however, this level was lowered sufficiently to allow the development of skin granulomata, six in the case of the partially thyroidectomized and three in the thymectomized rabbit. Since no further lesions developed it may be presumed that the plane of resistance again was raised, probably in connection with the occurrence and healing of the skin lesions. The disease picture of these two rabbits should be compared with the similar one of Control Rabbit 8 (Table III, Group D). In this animal there were two lesions of the bone and five of the skin but the resistance of this animal, unlike that of the partially thyroidectomized and thymectomized rabbits mentioned above, was not sufficient to prevent the subsequent development of a keratitis. In other words, the least resistant control animal was less resistant than the least resistant partially thyroidectomized and thymectomized rabbits.

There are several phases of this work which are significant from the standpoint of the biology of syphilitic infections. It is evident that the general character or severity of the disease at the time of the experiment is a factor which may influence such effects as are induced by the various operative procedures employed. Thus, when the severity of the disease is of average grade, as in the first experiment, the effect of complete or partial removal of the thyroid is striking, as is shown graphically in Table IV. Syphilitic lesions were much more pronounced in the group of rabbits in which the thyroid had been completely removed than in the control group. The effect of a partial thyroidectomy, on the other hand, was in the opposite direction, the disease being less severe than in the controls. When, however, the disease was pursuing a relatively mild course with fewer generalized lesions, as in the second experiment, the effects of complete or partial thyroidectomy were less conspicuous (Table V).

If one considers experimental syphilis of the rabbit as largely determined, in its manifestations, by the reaction and resistance of the host, either natural or acquired, then it is evident that animal resistance was on a higher plane in the second experiment than in the first. In such rabbits as those of the second experiment, factors which would operate toward a depreciation of resistance might not be able to produce this effect to the degree that would be possible under inherent or so called "natural" conditions of lower resistance. On the other

hand, factors which tend to increase the forces of defense or to render them more efficacious might be reflected in a disease little if at all milder than that which would actually develop in such initially resistant animals.

In this discussion of the relationship between the manifestations of the disease and the resistance of the experimental animal, one other point may be briefly referred to. It has been shown that the occurrence of various manifestations of the disease as well as their severity and duration bear a definite relationship to one another (6). Lesions of the eyes occur most frequently in cases of severe syphilis or in animals in which previous lesions have been comparatively slight or of relatively short duration; they rarely occur in animals that show a prompt and vigorous reaction. It may be added that eye lesions are usually terminal events in the manifestations of the disease, and that the most common type is a keratitis. Extensive cutaneous involvement, save in cases of malignant syphilis, is usually associated with minor bone lesions or none, and this condition has been interpreted upon the basis that the resistance of the animals, which was sufficiently high following the primary orchitis to prevent the development of bone lesions, was not sufficient to afford protection to the skin. The occurrence of bone lesions, however, indicates that the resistance of the animal is not high at the time of their development. If no cutaneous lesions develop, a sufficient resistance may be assumed to have taken place in association with the development and course of the preceding lesions.

The primary reaction of the completely thyroidectomized rabbits was unusually prompt and vigorous, but it was followed by numerous secondary manifestations including a large proportion of eye lesions. There were eight instances of eye involvement among four animals of each experiment. Among the partially thyroidectomized rabbits, on the other hand, although the primary reaction was, in general, slight, the subsequent course of events was contrary to what might have been expected in normal animals. In the first experiment there was only one lesion of the bones and two of the skin, but instead of a fair number of eye lesions subsequently only one developed, indicating the persistence of a high state of resistance. In the second experiment there was again one bone lesion but seven of the skin, six of which

occurred in one animal,—the two instances of eye lesions developed in the animal that had previously a single granuloma of the skin. While the proportion of eye lesions in the partially thyroidectomized rabbits of the second experiment is practically the same as in the controls, they were distributed in the control group among four animals, an animal incidence of 50 per cent, whereas in the operated group they were limited to one, an animal incidence of 20 per cent.

In the thymectomized group, somewhat different conditions prevailed. The primary reaction had a closer resemblance to that of the controls than to that of the partially thyroidectomized rabbits, but the generalized manifestations were less marked especially as regards animal incidence. However, there was only a single instance of eye involvement in the thymectomized group.

The percentages for the proportion of eye lesions which developed in the several groups of both experiments, figured upon a basis of possible numbers, is as follows: complete thyroidectomy, 61.5 per cent; partial thyroidectomy, 13.6 per cent; thymectomy, 8.3 per cent; controls, 21.4 per cent. In other words, a high proportion of eye lesions in a group of animals with other syphilitic lesions of considerable severity indicates a state of low resistance, while few eye lesions in rabbits with other manifestations of a minor character indicates a high level of resistance.

It is significant that the operative procedures employed did not produce identical effects in every rabbit of a series; that is, individual variations in the clinical manifestations of the disease occurred as in normal animals. It would appear, therefore, that our interference with the thyroid or thymus glands merely altered the reaction of the host on whatever plane or in whatever state it happened to be. From one standpoint the most outspoken effect was obtained with complete removal of the thyroid. This tended in the direction of lowered resistance resulting in a more severe disease. In the case of a malignant neoplasm with which we have worked (2) this effect practically always occurred and was very striking. The less constant effects in syphilitic infections are probably to be explained by the difference in type of the two diseases. In normal rabbits the neoplastic disease is acute or subacute and a certain proportion of deaths occur from widespread metastatic involvement as early as 3 to 4 weeks after

inoculation; while experimental syphilis of the rabbit is essentially a chronic condition, in which secondary lesions in remote parts of the body do not usually occur for about 2 months and which, in the great majority of cases, tends toward complete recovery within 4 to 6 months. Thus, in the case of experimental syphilis there is an opportunity for physiological readjustments between the time of inoculation and the development of generalized lesions which is not present in the case of the tumor. If, on the other hand, such readjustments or compensations are not forthcoming in the case of a syphilitic infection, the more marked generalized manifestations of this disease are comparable to the higher incidence and widespread distribution of metastases seen in the malignant disease of thyroidectomized rabbits. That the factor of time enters in is brought out by the character of the primary and metastatic orchitis, which in both of the experiments here under consideration were more pronounced in the completely thyroidectomized groups than in the controls.

There is some indication that even individual animal variation may have been influenced to some extent by our procedures. The infection was in no instance more than of slight or fair grade in the instances of partial thyroidectomy and perhaps also in those of complete thymectomy, whereas in the controls of the first experiment half of the rabbits had a moderate or well marked infection and in the second experiment there was one such instance (Table II). While it is not impossible that all the rabbits in the partially thyroidectomized and thymectomized groups were naturally of high resistance, the chances are that at least one in each group was less resistant than the others and if not interfered with would have developed a moderate or well marked grade of infection.

It would appear from these experiments that, in the rabbit, surgical removal of the thyroid and of the thymus is followed by alterations in the clinical manifestations of experimental syphilis. We interpret such alterations as a consequence of changes in the mechanism of the host's reaction or resistance to the infection. From this point of view the experiments furnish additional evidence in support of the conception referred to in the beginning of this paper, that the integrity and balance of the system of glands of internal secretion play an important part in the reaction of the host to disease conditions.

SUMMARY AND CONCLUSIONS.

Experiments are described in which the thyroid or thymus gland of rabbits was removed prior to inoculation of the animals with *Tr. pallidum.* The effect of these procedures is described from the standpoint of the manifestations of the disease. After complete thyroidectomy, the disease was considerably more severe than in the controls and very markedly so in certain instances. Partial thyroidectomy, on the other hand, resulted in a milder disease than that of the controls. The effect of complete thymectomy was less pronounced than that of either complete or partial thyroidectomy, but, in general, the syphilis resembled that in partially thyroidectomized animals.

These effects are discussed in relation to the host's reaction and resistance to experimental syphilis and the conclusion was reached that the integrity and balance of the glands of internal secretion play an important rôle in the mechanism of defense against this infection.

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OBSERVATIONS ON THE HUMAN BLOOD GROUPS.

Irregular Reactions. Isoagglutinins in Sera of Group IV. The Factor A[1].

By K. LANDSTEINER and DAN H. WITT.

(From the Laboratories of The Rockefeller Institute for Medical Research and the Second Medical (Cornell) Division of Bellevue Hospital, New York.)

The present paper deals with the individual differences of human blood, as manifested by agglutinin reactions, especially with the occurrence of exceptions to the rule of the blood groups (Landsteiner) (1), (2), (6). In the practical application of the isoagglutinin tests in blood transfusion which has been suggested by one of the present writers (2), these irregularities are usually a matter of minor consequence, for in most cases it will be possible to carry out direct cross-tests of the blood of the donor, and the recipient, in addition to the grouping, and this procedure is obviously and for several reasons the proper method. But there are other reasons which justify a detailed consideration of the subject. It is intimately connected with the theoretical question of individual bio-chemical specificity and since the work of v. Dungern and Hirschfeld (3), L. and H. Hirschfeld (4), Ottenberg (5), with the problems of human inheritance and racial differences in man. Such a study is also desirable because of the contradictory views put forth recently, some of which might confuse the reader not intimately acquainted with the subject.

The occurrence of irregular isoagglutinin reactions, if cross-tests are made between a number of blood samples, has been mentioned long ago (2), (6). Several of the observations recorded since are certainly due to technical errors which can occur if the tests are made on slides and the readings are taken after a short time. In this way slight reactions may be overlooked in view of the fact that the agglutinative strength differs considerably in various sera.[1] Also in

[1] A higher titer may possibly occur in pathological conditions (1), (8), (9).

group I sera the relative titers for II and III blood vary individually. Examples of this possibility of faulty typing are given by Dyke (10) and Brem (11). To avoid mistakes and particularly in doubtful cases a small amount of diluted washed blood emulsion should be used, the tests set up in tubes, and watched for a sufficient length of time.

Pseudo-Agglutination. Auto-Agglutination.

Causes of error in the opposite direction leading to false positive results are the auto-agglutination (cf. Hooker and Anderson, 12a) and another phenomenon, namely, the rouleaux formation and agglomeration of red cells connected with the increase in the rate of sedimentation of certain bloods (human blood in pregnancy, infectious diseases, normal horse blood, etc.). These facts, known for a long time, have been studied assiduously of late, following the work of Fahraeus. That this phenomenon, which may be termed pseudo-agglutination (9), can be and has been mistaken for iso-agglutination is exemplified by the observations of Shattock (12). Even though this author clearly states that his reactions could only be observed in pathological cases, and emphasizes the coincidence of it with the rapid subsidence of blood and the production of a buffy coat in inflammatory conditions his observations were frequently quoted—also by ourselves—as instances of iso-agglutination. That the agglomeration is largely inhibited by slight dilution—as it was in Shattock's experiments—is a point which discriminates between pseudo-agglutination and "real" agglutination. He remarks,[2] "The blood serum, however, stands very little dilution. If one loop of salt solution is mixed with one of the serum, and to one loop of the mixture is added one of normal blood, the typial picture no longer presents itself, the hanging drop not being appreciably different from that of normal blood."

The active substance of the serum which causes pseudo-agglutination and rapid sedimentation of the erythrocytes cannot be absorbed like agglutinins by these cells. This is another feature distinctive between pseudo- and any "real" agglutination (K. Meyer and Ziskoven, 13; Lattes, 14; Mino, 9; Landsteiner and van der

[2] L. c. page 311. See also p. 313.

Scheer, 15). Pseudo-agglutination is, as a rule, not less, but may be even more pronounced at 37° than at a lower temperature. A picture similar to that of pseudo-agglutination and an increased rate of sedimentation can easily be obtained if to blood suspensions certain colloidal substances like gum arabic or gelatin are added.

Auto-agglutination has been characterized by one of the present writers (16), as follows: It occurs, as a rule, with the blood of animals —for instance horse or rabbit blood, or the blood of man (cf. Mino), when erythrocytes are added to a sufficient amount of serum of the same individual and the mixture kept at low temperature, as in the ice-box. The reaction is greatly diminished by increasing the temperature, much more than the effect of hemagglutinins is in general, so that in most cases it disappears completely at 37°. The active substance is absorbed by red cells at low temperature and can be separated from them if the agglutinated corpuscles, after washing with cold saline, are slightly warmed in saline solution, e.g., kept at room temperature. The rate of sedimentation and the intensity of auto-agglutination do not run parallel in various samples of individual human bloods (personal observations). In human blood the intensity of the latter varies and is sometimes increased in pathological cases. In certain diseases exceptionally strong reactions have been observed, as in syphilitic or hypertrophic cirrhosis, paroxysmal hemoglobinuria, hemolytic icterus, anemia, trypanosomiasis and spirillosis both of man and animals (6, 17. 18, 19, 20, 21, 22, 23). Rous and Robertson (24) induced the production of powerful auto-agglutinins in rabbits by repeated bleedings or transfusions with rabbit blood.

The auto-agglutinins react not only on the cells of the blood from which the serum was taken but also on those of other individuals of the same species and obviously, under certain conditions, they may simulate iso-agglutinin reactions. It will be seen, however, that exceptionally a sharp line cannot be drawn between the two sorts of reactions.

Irregular Iso-Agglutinin Reactions.

A number of the observations on iso-agglutination which are at variance with the common rule are incidental ones, not thoroughly

studied, owing, presumably, to the difficulty of securing human material. It is hardly justified to draw any conclusions from such casual observations as certain ones quoted by Guthrie and Huck (25), before similar cases are encountered, as, for instance, to grant as established the existence of bloods which are agglutinated by practically any serum I and not by the sera II and III.

The findings can be grouped under several headings.

Sera with Deficient Reactions.

In several communications (26, 27, 28) bloods have been described, the corpuscles of which behaved in the regular way, while the sera gave fewer reactions than were to be expected from the rule of groups, sera of group I (to judge from the red cells) for example, agglutinating only blood III, but not blood II or sera of group III not active for blood II. In some of these observations weak reactions very probably have been overlooked. The use of non-washed blood may, perhaps, tend to increase this possibility on account of inhibiting substances present in the serum (see Moss). In other cases, however, there is no reason to doubt that there was really a deficiency of the iso-agglutinins (Sucker, 28a). Since the amount of agglutinin present in the serum may vary from time to time (Dyke (10) and others), there exist, possibly, instances in which the lack of agglutinins is not permanent. The regular occurrence of such a condition in infants is known from the work of v. Decastello and Sturli (29) and Happ (30).

Strong Anomalous Reactions.

Examples of strong, entirely irregular reactions are evidently very scarce, and the cases reported have not been studied thoroughly enough.

We are indebted to Dr. R. Ottenberg for the knowledge of an unquestionable case which is in course of publication. In this instance the serum contained an anomalous agglutinin capable of acting on the cells of all the blood groups, and which was sufficiently strong to lead to a fatal hemoglobinuria after transfusion.

In a case of Sucker (28a) the corpuscles behaved like blood II;

the serum like that of group III. No absorption experiments were made, and it is not stated how many different sera and corpuscles were used for the tests.

Slight Atypical Reactions. "Cold" Agglutinins.

Unger (31)[3] made the following statement: " . . . . I have occasionally noted that although the donor and patient are of the same group, when the bloods are tested one against the other a small number of agglutinated clumps of red cells will be seen." "Agglutination of cells by sera of the same group is probably due to a 'para' or 'minor' agglutinin which causes a lesser degree of agglutination." Unger believes that the phenomena described are important for the effect of transfusion (cf. Hooker and Anderson). Observations similar to those of Unger are recorded by other authors (see 32). Some results of ours, resembling those of Unger, are shown in tables 1 and 1a.

Technic.—One drop of serum, one drop of saline, one drop 2.5 per cent washed blood emulsion were put in small test tubes with a diameter of about 7 mm. The tubes were occasionally shaken. For the readings a small drop was examined on a slide under the microscope. (The same technic has been used throughout the experiments described in the present paper, unless special mention is made.)

Most of the blood samples used throughout this study were taken from patients in the hospital, only a few having been taken from normal individuals.

The reactions presented have the peculiarity of being markedly lessened by a slight increase in temperature, and this fact suggests a relationship to the reactions described as auto-agglutination.[4]

While observations like those presented in tables 1 and 1a are exceptional, it is a common occurrence, as mentioned above, that at a temperature not much above 0° human serum causes agglutination, not only of the red cells of the same, but also of other individuals, the effect being diminished by increased temperature, and depending on the amount and the activity of the serum. As experiments by Bialosuknia and Hirszfeld (33) tend to show, the strength of these

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[3] See Culpepper and Ableson. Journ. of Lab. and Clin. Med. **6.**
[4] See Unger, L. c., p. 11.

reactions varies if different kinds of corpuscles are tested (see 33a). The specificity disclosed in this way is independent of that of the typical iso-agglutination. Our own experiments seem to confirm this view, as the experiments presented in table 2 show.

<div align="center">TABLE 1.</div>

Weak Irregular Reactions within the Same Blood Group under Approximately Routine Conditions (Low Room Temperature). Reading after One Hour at 18°C. The Strength of the Reaction Is Indicated as Follows: F.Tr. = Faint Trace; Tr. = Trace; ±, +, + ±, etc. The Roman Figures Indicate the Groups.*

SERA	CORPUSCLES						
	1_I	2_I	3_{II}	4_{II}	5_{III}	6_{III}	7_{III}
1_I	0	0	+++	+++	++	++	++
2_I	0	0	+++	+++	++	++	++
3_{II}	0	0	0	0	++	++	++
4_{II}	0	0	0	0	+++	++	+++
5_{III}	0	0	+++	+++	0	0	0
6_{III}	0	0	+++	+++	0	0	+†
7_{III}	0	0	++	++	0	0	0

<div align="center">TABLE 1a.</div>

SERA	CORPUSCLES					
	8_I	9_{II}	10_{II}	11_{II}	12_{III}	13_{III}
8_I	0	+	+	+	+	+
9_{II}	0	0	0	0	+	+
10_{II}	0	0	0	±†	++	++
11_{II}	0	0	0	0	++	++
12_{III}	0	++	+	++	0	0
13_{III}	0	+++	+++	+++	0	0

* Nomenclature of the American Committee, Jour. Amer. Med. Assoc., 1921, lxxvi, 130..

† Weak atypical reactions.

Bialosuknia and Hirszfeld are inclined to separate this kind of reaction from true iso-agglutination, while Guthrie and Pessel (32, 33) favour the opposite view. In tables 3a, 3b, 3c some of our experiments concerning this question are summarized.

Technic.—To 0.5 cc. of progressively doubled dilutions of the serum one drop of 2.5 per cent blood emulsion was added. Sera containing distinctly active cold

TABLE 2.

Effects of Cold Agglutinins. Tests Kept Overnight in Ice-Box; Read at 0°. For the Sake of Clearness, the Common Iso-Agglutinin Reactions, Which Were Typical, Are Not Recorded, but the Roman Figures Indicate the Groups.

SERA	CORPUSCLES									
	90_I	91_I	92_I	93_I	94_{II}	95_{II}	96_{II}	97_{III}	98_{III}	99_{IV}
92_I	0	0	0	0						
93_I	+	+	±	±						
94_{II}	±	+	±	±	±	±	0			
95_{II}	+++	+++	+++	+++	+++	+++	+++			
96_{II}	++	++	++	++	++	++	±			
97_{III}	+	+	++	±				±	±	
98_{III}	±	tr.	+±	+±				±	±	
99_{IV}	±	+	+	+	+	++	±	+	+	+

TABLE 3a.

Serum 30 (I) Diluted 1:2 for the Tests with Blood 26 (I), 27 (I); Diluted 1:10 for the Tests with Blood 20 (II), 22 (III). One-Half Cubic Centimeter of Each Dilution Was Mixed with One Drop of 2.5 Per Cent Cell Suspension.

BLOOD NUMBER	DILUTION OF SOLUTION									
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256		
26_I	0								One hour at 22°	Typical iso-
27_I	0									agglutina-
20_{II}	+±	+	+	±	tr.	0				tion
22_{III}	±	+	+	±	0					
26_I	+	+	+	±	±	tr.	f.tr.		Same tests over-	Showing
27_I	+	+	+	+	±	±	tr.	0	night in ice-box	"cold" ag-
20_{II}	+±	+±	+	+	+	tr.	0		read at 0°	glutina-
22_{III}	+	+	+	+	±	tr.	0			tion
26_I	0								Same tests after	Typical iso-
27_I	0								one additional	agglutina-
20_{II}	+±	+	+	+	f.tr.	0			hour at 37°	tion
22_{III}	+	+	+	±	0					

agglutinins were selected. The tests were kept in the ice-box overnight. The tubes were occasionally shaken during the experiments.

TABLE 3b.

Serum 33 (II) Diluted 1:2 for the Tests with Blood 33 (II), 34 (II); Diluted 1:10 for the Tests with Blood 35 (III).

BLOOD NUMBER	DILUTION OF SOLUTION					
	1:2	1:4	1:8	1:16	1:32	
33_{II}	0					One hour at 22°
34_{II}	tr.	0				
35_{III}	+±	+	±	f.tr.		
33_{II}	tr.	f.tr.	0			Same tests overnight in ice-box read at 0°
34_{II}	+	+	±	0		
35_{III}	+±	+	±	f.tr.	0	
33_{II}	0					Same tests after additional two hours at 21°
34_{II}	±	tr.	0			
35_{III}	+	±	tr.	0		
33_{II}	0					Same tests after one additional hour at 37°
34_{II}	0					
35_{III}	+	tr.	f.tr.	0		

TABLE 3c.

Serum 34 (II) Diluted 1:2 for the Tests with Blood 33 (II), 34 (II), 37 (I); Diluted 1:10 for the Tests with Blood 35 (III).

BLOOD NUMBER	DILUTION OF SOLUTION						
	1:2	1:4	1:8	1:16	1:32	1:64	
33_{II}	0						One hour at 22°
34_{II}	0						
37_{I}	0						
35_{III}	+	+	+	±	tr.	0	
33_{II}	+	+	±	±	f.tr.	0	Same tests overnight in ice-box read at 0°
34_{II}	+	±	tr.	0			
37_{I}	+±	+	+	+	±	tr.	0
35_{III}	+±	+	+	+	±	0	
33_{II}	f.tr.	0					Same tests after additional two hours at 21°
34_{II}	0						
32_{I}	tr.	0				0	
35_{III}	+	+	+	±	tr.		
33_{II}	0						Same tests after one additional hour at 37°
34_{II}	0						
37_{I}	0						
35_{III}	+	+	±	±	0		

The experiments show again that the substances active at low temperature may influence individual corpuscles, also of the same group, to a different degree. As the temperature is elevated the "cold"-agglutination tends to disappear. Some reactions may persist longer than others, and thus give the appearance of minor agglutinations such as those presented in tables 1 and 1a.

For the present a sharp line is not to be drawn between auto-agglutinins and "cold" iso-agglutinins (Panhaemagglutinins of Mino), and it will be seen later that there exist also transitions between the latter and common iso-agglutinins. Nevertheless, there is, as a rule, a striking difference between the "cold" iso-agglutinins and the common iso-agglutinins with regard to their strength and the way in which they are influenced by changes in temperature. The reactions of the sera II, Nos. 33 and 34 on blood III (tables 3b and 3c), for example, have about the same intensity when read after one hour at 22° and at 0° after standing in the ice-box overnight; their reactions on blood of the same group, on the other hand, are very marked at 0°, and almost absent at 22°, even though an unusually large quantity of serum was used. They would have escaped detection under the conditions of routine technic. The differences are thus great enough to warrant a discrimination. If tests are made under ordinary conditions the rule of the four groups holds remarkably well, and is a fair approximation. Therefore, it seems hardly advisable, as Guthrie and Huck suggest, to relinquish this classification. The reactions occurring at low temperature and several other anomalous agglutinations have not yet been studied sufficiently to be included in a practicable classification. An analysis of the agglutination at low temperature can probably be made by absorption experiments (see 34, 35) which may possibly reveal the existence of several "cold" agglutinins in one serum.

Agglutinins in Group IV.[5]

V. Dungern and Hirschfeld (37) reported that after absorption of a group III serum with certain group II corpuscles the resulting

[5] One of the present writers (Landsteiner) has been repeatedly criticized since a paper published by Moss (36) for having made an incorrect statement recog-

fluid still acted on some corpuscles of group II but not on others of the same group. On account of these results they suggested a subdivision of group II. Similar observations were communicated by Schütze (38), Guthrie and Huck (25), Coca and Klein (39). The reactions were explained by the assumption of an agglutinable factor in certain corpuscles II besides the factor A, and a corresponding additional agglutinin in sera I and III.[6]

In connection with these facts is an observation described by ourselves (40), concerning a serum IV (Pat. Barnett), containing agglutinins. Sera of group IV are generally characterized by the absence of agglutinins for any kind of human corpuscles. The corpuscles of the blood in question were agglutinated in the regular way by all sera I, II, and III. The serum, however, was found to contain agglutinins. The tests were made, as mentioned above, by mixing one drop of serum, one drop of saline, and one drop of 2.5 per cent washed blood emulsion.

At 0° agglutination occurred with practically every blood, but to different degrees. In tests made and kept for one hour at 15°, most of the reactions disappeared, and distinctly positive ones remained only for a number of bloods of group II, faintly positive ones for some other bloods. At a still higher temperature, (20°) that is, under conditions similar to those of routine tests, 18 bloods of group I and 8 of group III gave negative reactions, while 16 of 21 bloods of group II gave positive ones. Also one blood of group IV was agglutinated by the Barnett serum but not the corpuscles of Barnett. The question arose as to whether or not the agglutinin observed was to be identified with the factor A[1] detected previously in sera

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nizing only three types of human blood instead of four, and much attention has been paid this point. As a matter of fact the work of Landsteiner (1901) was pursued with his consent by Sturli, who had coöperated in Landsteiner's experiments, and Decastello and these authors (29) described the fourth blood group in 1902. Landsteiner (40) gave a complete description of the four blood groups in a monograph (6) (1909), which appeared before the paper by Moss.

[6] Instead of the designation Cc or xX, which have been proposed (25, 39) for this iso-agglutination pair we shall use $\alpha^1 A^1$, which indicate their association with the important α A pair. This is done regardless of the accuracy of the explanation which will be presently discussed.

of groups I and III.   With the object of investigating this point, comparative tests were made with the Barnett serum and a serum I (A. F. C.) absorbed first with a certain blood II (Lev.), not containing the supposed factor A^1 and then with a blood of group III.   A satisfactory agreement was found in the two series of tests.   The absorbed serum I did not react with the blood of Barnett, but with the other blood IV, indicating that the cells of Barnett did not contain the factor A^1, while the other group IV cells did.   The readings were made after one hour at 20° (table 4).   According to these results one may assume the existence of two sub-groups of group IV, the first being more common.

	IV(1)	IV(2)
Serum...	—	α^1
Corpuscles...	A, A^1, B	A, B

TABLE 4.*

SERA	CORPUSCLES OᵣF 9 INDIVIDUALS OF GROUP II								
Barnett..............................	+	±	±	tr.	tr.	±	0	±	0
A. F. C. absorbed with blood Lev. and a III blood..............................	+	±	±	±	±	+	0	+	0

* In the preliminary communication (40) the two lines of table 4 were erroneously transposed.

Another serum IV (Sn.) reacting in a similar way, but more intensely, has been found since our first report (40).

The blood Sn. was agglutinated by all sera II and III tested. A representative series of tests with the serum Sn. is given in table 5.

Altogether 73 bloods were examined with serum Sn. of which 24 belong to group I, 31 to group II, 12 to group III, and 6 to group IV. The reactions were wholly negative with all bloods I and III, distinctly positive with 24 of the 31 bloods II and with 5 bloods IV; negative with its own blood.

The samples of bloods II and IV which reacted markedly were those containing the factor A^1 according to tests made with an absorbed serum I.   Three of the 7 bloods supposed to contain no A^1 from the reaction with absorbed serum I reacted slightly.

Beside the bloods already described we have found some others with similar properties, although the agglutinins found were less active, that is to say, the reactions were distinct only at rather low temperatures.

Blood IV (L.). Corpuscles typical. The serum agglutinates (in tests made at 16°C.) very slightly 1 of three bloods of group I, none of two bloods of group III, distinctly 3 of five bloods of group II. The same three were agglutinated by serum Barnett.

Blood IV (H.). Corpuscles typical. The tests with the serum were kept overnight in the ice-box and read at 0°. It agglutinated faintly 2 of ten bloods of group I and 1 of two bloods of group III, markedly 10 of twelve bloods of group II, one blood of group IV, not the individual's blood. The bloods of groups II and IV were tested also with a liquid obtained by absorbing a group III serum with corpuscles of group II, not containing A^1. Positive reactions took place with the same bloods as those agglutinated by serum IV (H.). All the reactions of serum H. disappeared at room temperature.

The reactions with another serum IV (J.) were similar. Several bloods were agglutinated in tests kept overnight in the ice-box and then for one hour at 16°. The strongest reactions occurred with the bloods of group II containing A^1.

No attempt has been made to decide whether those agglutinins in these group IV sera which act on cells of the other groups can be absorbed and leave intact the agglutinin α^1, acting on cells AA^1.

The agglutinins found in the various sera IV lend support to the notion that there is not a sharp break between cold agglutinins and common iso-agglutinins. While the reactions of serum H. were manifest only at low temperature, those of serum Sn. were quite pronounced at 25° and even higher, like usual iso-agglutinin reactions of medium strength.

In two other cases "cold" agglutinins were observed in group IV sera which reacted with no recognizable regularity on different types of blood and also on the individual's corpuscles. Of the two sorts of bloods II those containing A^1 were in some cases rather less agglutinated than the other cells II in these observations.

On the whole, 30 sera of group IV were examined. Agglutinins acting chiefly on cells II AA^1 were encountered 5 times.

A short time after our communication on a serum of group IV containing iso-agglutinins, an apparently similar observation was reported by Lattes and Cavazzuti (41). The blood in their case was agglutinated by sera of groups II and III like a common group IV blood, the serum agglutinated most of the group II corpuscles,

but also two of 13 red cells of group I and 1 out of 5 samples of group III. Strong reactions, similar to common iso-agglutination, were obtained only with group II erythrocytes, corresponding to the

TABLE 5.

Reading after Two Hours at 20°.

Bloods....	GROUP													
	I	I	I	II A¹	II A¹	II	II A¹	III	III	III	III	IV A¹	IV A¹	IV
	236	237	239	238	241	242	244	233	234	235	240	243	245	Sn.
	0	0	0	+	+	0	+±	0	0	0	0	+	+	0

TABLE 5a.

Showing the Absorption of the Agglutinin from a Group IV Serum (Sn.). Absorption: To Serum Sn. 1/2 the Volume of Washed Sediment of Various Bloods Was Added, the Mixtures Were Kept Overnight in the Ice-Box and Centrifuged at 0°. Tests Read after One Hour and 30 Minutes at 24°.

	TESTED WITH BLOOD				
	Sn.	242	238	244	245
Group.....................	IV	II	II A¹	II A¹	IV A¹
Serum Sn......................	0	f.tr.	+	+±	+
Serum Sn. absorbed with blood Sn..............	0	0	±	+	±
Serum Sn. absorbed with blood 242 II A.........	0	0	tr.	+	tr.
Serum Sn. absorbed with blood 244 II A A¹.......	0	0	0	0	0

TABLE 5b.

Showing the Splitting Off of the Agglutinin Absorbed from a Group IV Serum. Tests Read after Two Hours at 21°.

	GROUP II	GROUP II A¹
Bloods.......................................	242	244
Fluid F.......................................	0	+

alleged variety AA¹. In absorption experiments the authors did not succeed in removing the questionable agglutinin from the serum by treating it with the sensitive red cells, and accordingly they concluded that they had not to deal with true iso-agglutination but with pseudo-

agglutination.   Indeed a certain difficulty arises for this supposition from the fact that there was a distinct degree of specificity in that the serum reacted by preference on the AA[1] cells.   Lattes and Cavazzuti tried to explain this phenomenon by assuming that the cells in question are in general more sensitive.   In our experiments, however, the results were different from those of Lattes and Cavazzuti.   With both the sera Barnett and Sn. the absorption experiments were successful and there was no difficulty in splitting off the agglutinins from their union with erythrocytes (see tables 5a and 5b).

When the absorption was made with a blood of the less sensitive sort (II A) by keeping the mixtures for one to two hours, not in the ice-box but at 20°C. no distinct diminution of its activity for the sensitive cells II was observed.

A separation of the agglutinin after its absorption with a sensitive blood II was made by adding 1 drop of packed, washed cells to 0.6 cc. of serum Sn., keeping the mixture overnight in the ice-box, centrifuging and washing at room temperature, and warming the aggultinated cells with 0.3 cc. saline at 56° for 3 minutes, till the clumps were broken up.   The mixture was then centrifuged in a jacket of warm water for a very short time, and the supernatant fluid was drawn off and centrifuged till clear.   The results of the tests with this fluid are shown in table 5b.

It appears that in our tests true iso-agglutinins were involved. Still the question remains as to the nature of these agglutinins. Mino (42) and Lattes and Cavazzuti (41) doubt the existence of the agglutinin α^1 and the agglutinogen A[1] first described by v. Dungern and Hirschfeld, and hold the view that the phenomena in question are due to purely quantitative variations in the pioperties of the corpuscles.   Experiments recorded by Lattes and Cavazzuti lend strong support to this belief, and should not be disregarded without further test as has been done by some authors (43).

The Agglutinin α^1.

The Italian writers mentioned lay stress upon two facts, namely, that the corpuscles AA[1] (more frequent at least among the North American whites) are more sensitive against sera I and III than are

the other corpuscles of group II and further that it is possible to absorb all agglutinins from a serum $\alpha\alpha^1$ by adding a sufficient amount of the corpuscles A. We have been able to confirm both of these significant observations.

TABLE 6.

Titration of Various Sera with Bloods A (50, Lev.), A A¹ (55, 58, 59) Using 0.2 Cc. of the Dilutions of Serum and 1 Drop of 2.5 Per Cent Washed Blood Emulsions. The Figures Indicate the Highest Dilutions at Which Agglutination Was Seen Microscopically. Readings after Two Hours at Room Temperature.

SERUM NUMBER	GROUP	BLOOD NUMBER				
		50	55	58	59	Lev.
57	I		160	240		80
52	I	40	160	80	160	40
53	I		16	16		4
51	III	15	60	40	40	15
67	III		20	40		5

TABLE 7.

2 Cc. of Serum A. F. C. (I) Treated for One Hour at Room Temperature with 1 Cc. of the Washed Sediment of Lev. Blood (II Containing No A¹) = Solution 1. Solution 1 Absorbed Again with 0.4 Cc. Lev. Sediment, Kept for One Hour at Room Temperature = Solution 2. To Solution 2 (about 1 Cc.) Added 0.4 Cc. Lev. Sediment, Kept Overnight in Ice-Box and Centrifuged in Ice = Solution 3. Bloods II. (A, A¹), 41, 42, 43, 44, 49, 51, Blood II (A) Lev. Readings after One Hour at Room Temperature.

SOLUTION NUMBER	BLOOD NUMBER						
	41	42	43	44	49	51	Lev.
1	+	+±	+	+±	+±	+±	0
2	+	+	±	+±	+	+±	0
3	tr.	tr.	0	±	±	tr.	0

The lesser agglutinability of the red cells A as compared with the cells AA¹ is shown in table 6.[7]

The experiment in table 7 shows that the blood Lev. (without A¹)

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[7] The bloods Lev. and A. F. C. are the same as those used in the work of Coca and Klein. For these and some other blood samples we are indebted to Dr A. F. Coca.

absorbs gradually most of the agglutinins, especially when the interaction takes place at low temperature.

In view of these findings it seemed necessary to investigate further whether the existence of the factor A^1 or the agglutinin α^1 can be definitely established or disproved. We tried to produce immune sera containing specific agglutinins for A^1 by injecting rabbits with

TABLE 8.

1½ Cc. Serum A. F. C. (I) Treated with 0.75 Washed, Packed Cells Lev. (A), by Adding Them in Four Portions during 1 Hour at Roqm Temperature (22°C.) and Centrifuging = Solution 1. 0.2 Cc. of Washed Blood Sediment Lev. Added to 1 Cc. of Solution 1 and Centrifuged after One Hour at Room Temperature = Solution 2. Serum A. F. C. Diluted 1:10 = Solution 3. 1 cc. Serum A. F. C. Diluted 1:10 Absorbed with 0.2 Cc. of the Sediment Lev. = Solution 4. All the Solutions Were Titrated as Above and Read after One Hour at Room Temperature. The Figures Refer to the Total Dilution of the Serum.

DILUTION	BLOODS				DILUTION	BLOODS			
	No. 74 A A¹	No. 79 A	No. 84 A A¹	Lev. A		No. 74 A A¹	No. 79 A	No. 84 A A¹	Lev. A
	Solution 1					Solution 2			
1:1	+±	0	+	0	1:1	+	0	+	0
1:2	+	0	+	0	1:2	+	0	+	0
1:4	±	0	±	0	1:4	±	0	tr.	0
1:8	0	0	f.tr.	0	1:8	0	0	0	0
	Solution 3					Solution 4			
1:10	+±	±	+	±	1:10	tr.	0	tr.	0
1:20	±	0	tr.	0	1:20	0	0	0	0
1:40	f.tr.	0	0	0	1:40	0	0	0	0

blood corpuscles AA^1 and absorbing them with erythrocytes A in order to remove the agglutinin α. An analogous method (Landsteiner (44), Hooker and Anderson (45)) has proved very useful for determining the factors A and B, and Huck and Guthrie (46) maintain that they succeeded in preparing immune sera specific for A^{1}.[3]

Our attempts to produce such immune sera did not yield clear-

[3] In the paper by Huck and Guthrie the figures 0–304 and 0–480 apparently do not take into account the dilution 1:8 of the immune sera employed for the absorption.

cut, positive results. The solutions obtained were not distinctly different from similar ones prepared from normal rabbit serum. Possibly this failure is due to accidental circumstances. Various normal rabbit sera behave differently. Some of them were less active for cells A than for AA¹, and in absorption experiments they resembled human sera I or III.

TABLE 9.

Demonstrating the Separation of the Iso-agglutinins α and α^1.

SOLUTION NUMBER	BLOOD	DILUTION OF THE SOLUTION										
		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:178	1:256	1:512	
1 {	A A¹	+++	++	++	++	+±	+	+	±	tr.	0	One hour at 25°
	A	+±	+±	+	+	+	±	±	tr.	0		
2 {	A A¹	+	+	+	+	+	±	tr.	f.tr.	0		
	A	0										
1 {	A A¹	+++	+++	+++	++	++	+±	+	±	tr.	0	Same tests overnight in ice-box read at 0°
	A	++	++	+±	+±	+	+	+	tr.	0		
2 {	A A¹	+±	+	+	+	+	+	tr.	tr.	0		
	A	±	tr.	0								
1 {	A A¹	+++	+++	++	++	+±	+	+	±	tr.	0	Same tests after thirty minutes more at 25°
	A	+±	+±	+±	+	+	+	+	tr.	0		
2 {	A A¹	+±	+	+	+	+	±	tr.	f.tr.	0		
	A	0										

Analogous experiments were made with similar results with another serum III and two pooled sera I.

According to the hypothesis of the Italian authors the absorption of a serum I or III with cells A should only *diminish* their content in agglutinins and it should thus be possible to prepare dilutions of a serum I or III, which are indistinguishable from the fluid prepared by treating the same sera with the less sensitive cells A. However, this seems not to be the case.

One sees from the experiment presented in table 8 that in the first place the titers of the absorbed serum for blood AA¹ and A respectively

were more different than the titers of the same serum merely diluted. Secondly, the activity of the latter was more diminished by treatment with cells A for cells AA[1] than the titer of the serum absorbed previously.

In experiments of another sort which gave more conclusive results, the following method was adopted (table 9):

To 8 cc. of a serum III were added 0.25 cc. of washed sediment of a blood II A (containing no A[1]). The mixture was centrifuged after two hours standing at room temperature, the sediment was washed twice with ice-cold saline. Then, to the sediment, 0.7 cc. saline was added, the mixture was heated at about 55°C. for 5 minutes or less, shaken several times and centrifuged as in the experiment (page 216). This fluid (solution 1) was prepared in order to obtain a solution of the agglutinin A. The fluid resulting from the treatment of serum III with blood II A was then absorbed with 4 cc. of sediment of the same blood for two hours at room temperature, in order to remove the agglutinin A entirely. After centifuging to the supernatant fluid 0.2 cc. of a blood II AA[1] was added, and after standing at room temperature for two hours an agglutinin solution was prepared by heating the sediment with saline as above. This solution (solution 2) was supposed to contain the agglutinin A[1]. During the digestions the mixtures were shaken several times. The fluids 1 and 2 were titrated in the manner described above with two bloods A A[1] and A, respectively.

The experiments were repeated with analogous results with other sera I and III.

The experiment shows that it is possible to obtain two kinds of agglutinating fluids, differing greatly from each other in their power of agglutinating the two kinds of group II cells. This fact leads to the conclusion that there exist two fractions of agglutinins with different properties in the sera I and III. As regards the corpuscles, the simplest assumption is apparently to ascribe two agglutinogens, that is, A and A[1] to the more sensitive cells II and only A to the others. From this point of view one must suppose according to the absorption experiments that agglutinin α^1 is to a slight degree capable of combining also with the agglutinogen A. On the other hand, it is possible to presume that the two kinds of red cells occurring in group II contain different agglutinogens, the one sensitive chiefly to α, the other to α and α^1. This uncertainty as to the actual state of affairs is due to the fact that with the available technical methods only the agglutinins can be separated, not the agglutinogens.

A similar situation exists, however, in most if not all absorption experiments carried out with agglutinins. In this respect it should be mentioned that, as Coca and Klein have pointed out, the absence of A^1 in a blood II does not imply the existence of α^1 in the serum, as could be expected if α^1 was quite analogous to α and β.

The agglutinin α^1 was present in all sera I and III examined by us (cf. Coca and Klein, 39), but we did not investigate this point completely.

From the work of Guthrie and Huck (25) it would seem that there exist sera III possessing agglutinins only of the type α^1.[9] The same holds apparently also for the agglutinin containing sera IV.

Regarding the other instances in which differences were found between the properties of individual sera and bloods of the same group (cf. 32, 34, 35, 46), the opinion of Mino and Lattes and Cavazzuti can hardly be contradicted that for every case thorough quantitative studies are needed in order to prove the existence of new iso-agglutination elements.

Individual Differences of Human Blood Demonstrable by Sera of Animals.

The main human iso-agglutinogens A and B are to be detected not only by human serum but also by the sera of animals (44, 26, 45). The method to be used consists in absorbing the normal or immune sera with one sample of corpuscles, and testing the resulting fluids with the red cells of other individuals. According to v. Dungern and Hirschfeld many individual differences can be found in this way in addition to those underlying the division into four groups (see 48). The belief that there exists a great number of bio-chemical individual variations of cells within a species is substantiated serologically by some tests made on the blood of cattle (49) and of chickens (50), and in general by the known facts concerning transplantation. Certainly the interpretation of the serological tests mentioned presents some difficulties as to whether the differences are of quantitative or qualitative nature. But experiments made by ourselves are also strongly suggestive of individual differences, other than those based upon the factors A and B.

[9] The tests were made at 37°, not under ordinary conditions (46).

Sera of ten cats were inactivated for one-half hour at 55°, then pooled. To portions of 5 cc. of the pooled sera 5 cc. washed sediment of various human bloods was added. The mixtures were allowed to stand in the ice-box overnight, and were centrifuged. A second absorption was made in the same way, the centrifugation being carried out after the mixtures had stood for two hours. For the tests 0.4 cc. of saline and 1 drop of 2.5 per cent washed emulsion of various human bloods were added to 0.4 cc. of the supernatant fluid. Readings were made after 1 hour at room temperature.

The protocol of this experiment is presented in table 10.

TABLE 10.

SERUM ABSORBED WITH CORPUSCLES	CORPUSCLES									
	151_I	155_I	156_I	157_I	159_I	152_{II}	158_{II}	153_{III}	154_{III}	160_{III}
151_I	0	0	0	0	0	++	+++	+	+	+
155_I	+	0	0	0	±	++±	++±	+	+	+
156_I	+	0	0	0	0	+++	++±	+	+	+
157_I	+	0	0	0	0	++±	+++	+	+	+
159_I	+		0	0	0	++±	++±	+	+	+
152_{II}	+	±	±	tr.	tr.	0	0	+	+	+
158_{II}	+	tr.	0	0	0	0	0	+	+	+
153_{III}	+	±	0	tr.	tr.	++	++±	0	0	0
154_{III}	+	0	0	0	0	+++	++±	±	0	0
160_{III}	+	±	tr.	±	+	++±	++	+	+	0

It appears from the tests in table 10 that nine of the ten blood samples can easily be classified in three groups, in accordance with the typing by iso-agglutination. The three bloods III did not react quite identically; particularly after the absorption with blood 160, agglutinins were left over for bloods 153 and 154. The reactions of the two bloods II were alike. Of the five samples of group I, four showed slight variations, and one (151) differed very markedly. Agglutinins for this blood remained after the absorption with any of the other bloods. These findings recall the interesting results of Hooker and Anderson with rabbit immune serum, which indicate the existence of an agglutinable factor in a blood of group I not present in the cells of the other groups. The value of our tests is increased by the fact that the results were essentially the same after the first

and the second absorption. It is to be noted, however, that other experiments made according to the same technic, but with other sera and blood, did not regularly show well-pronounced differences outside of the common blood groups.

In similar tests with pooled horse serum it was seen that after treatment with a certain blood IV the resulting fluids reacted on most other cells tested belonging to all four groups.

The Iso-Agglutination Elements.

It has been pointed out by one of the writers (2) that the iso-agglutinin reactions of human blood can possibly be explained by the simple assumption of only two different agglutinogens and agglutinins. Designating these by α and β, and the agglutinogens by A and B, the following symbols are obtained for the blood groups: I$-$ α, β; II A, β; III B, α; IV A, B $-$; if we include the factors A^1 and α^1 in the scheme, and if O and o signify the absence of agglutinogens or agglutinins, then the signs are: I O α, β, α^1; II A, β, and A, A^1, β; III B, α, α^1; IV; A, A^1, B, o; A, B, (α^1). Moss (36), confirming in his paper the facts concerning iso-agglutination previously established, suggested two new alternative explanations; the first, which Moss himself considers as less probable, would suppose that corpuscles IV do not absorb the agglutinins for cells II and III, and is therefore not acceptable; according to the second hypothesis the agglutinins of group I should be entirely removed by treatment with cells of any other group. Since this postulate was not borne out by the experiments of Koeckert (51), Schütze (38), Hooker and Anderson (45), Dyke (10), these authors rejected the theory of Moss. According to Hektoen (52) corpuscles II and III absorb the agglutinins of sera I for both these groups.

We studied the results of absorption experiments as well as the action of purified agglutinin solutions (53). These were prepared as described on page 220.

The agglutinin solutions prepared from sera II and III were found to agglutinate only corpuscles III and II respectively. On the other hand, the solutions made from sera I with either II or III cells were in most cases active for both kinds, though the one used for the preparation was affected more intensely.

Our absorption experiments were in conformity with these results, in so far as after the absorption of serum I with a blood II or III there resulted often, but not regularly, a considerable loss of activity for bloods III or II respectively.

One could conclude from these results and similar ones, to be found in the communications of the authors referred to, that the agglutinins a and b in sera I can be present to a certain degree in some sort of combination which corresponds to a formula for blood I as follows: O, α, β, $\alpha + \beta$.[10] The experiments, however, do not substantiate the hypothesis of Moss.

A certain relationship between the factors of cells II and III is shown by experiments of Hooker and Anderson and Doelter (45, 57), by the group-specific flocculation (56) and complement fixation (57) with alcoholic extracts of human red cells.

Strong evidence of this sort is afforded by experiments with the sera of chickens immunized with human blood of groups II and III. In view of the high titer of the sera it is very probable that they contained immune agglutinins. After absorption of these sera with group I cells a large amount of agglutinins was left behind for both group II and group III cells (table 12).

There are other facts which indicate that the usual scheme, though suitable for ordinary purposes, is not a complete representation of all known facts concerning human blood groups. It has been found that substances related to the human iso-agglutinins (44, 45) and iso-agglutinogens (26, 45, 55) occur in animals, but while, for example, rabbit cells absorb agglutinin β from normal human sera—the effects varying in degree in individual sera—they are not susceptible to the action of agglutinins, acting on B, in normal or immune rabbit sera. It is plausible that the rabbit sera lack such substances which would affect the cells of the animal itself. The two sorts of agglutinins—in human and rabbit sera—are therefore not identical.[11] Immune sera obtained from chickens after injections of human blood of group III, however, have a strong agglutinative effect on animal

[10] With regard to a genetic association of the iso-agglutination elements see the remark of Tebbutt and McConnel (54) on a possible linkage of A and B.

[11] Experiments made in coöperation with C. P. Miller in course of publication.

TABLE 11.

Instances of Absorption Out of a Group Serum of Iso-Agglutinins by Corpuscles Unrelated to Those Agglutinins. ½ Cc. of the Sera Were Treated with One-Half the Volume of Washed Blood Sediment for Three Hours at Room Temperature. The Original and the Absorbed Sera Were Titrated for Bloods II and III.

	BLOOD	DILUTION					
		1:10	1:20	1:40	1:80	1:160	1:320
Serum 375ᵣ........................	II	+±	+±	+	±	0	
Serum 375 absorbed with blood III....	II	+	±	0			
Serum 375........................	III	+±	+±	±	f.tr.	0	
Serum 375 absorbed with blood II....	III	+	±	0			
Serum 363ᵣ........................	II	++	+±	+	+	0	
Serum 363 absorbed with blood III....	II	+±	+±	+	tr.	0	
Serum 363........................	III	+±	+	tr.	0		
Serum 363 absorbed with blood II.....	III	+±	+	tr.	0		

TABLE 12.

Showing a Relationship between the Iso-agglutinable Substances of Cells II and III.

Chicken immune sera diluted 1 to 40 were absorbed with half the volume of washed human blood sediment for two hours at room temperature and overnight in the ice-box. The original and absorbed solutions were titrated. ½ Cc. of each dilution was mixed with one drop of 5 per cent cell suspension. Readings were made microscopically after two hours at room temperature.

CORPUSCLES OF GROUP	UNABSORBED	AFTER ABSORPTION BY CELLS OF GROUPS		
		I	II	III
Antiserum for group II cells				
I	200	<40	<40	<40
II	800	640	<40	160
III	400	320	40	<40
Antiserum from group III cells				
I	8000	<40	80	40
II	6000	2400	<40	<40
III	8000	3200	3200	<40

erythrocytes, sensitive for the human iso-agglutinin β, as those of the dog, pig, etc.

It may be mentioned that the resemblance between the factor A and Forssman's heterogenetic antigen, detected by the interesting experiments of Schiff and Adelsberger (58), and other observations (v. Dungern and Hirschfeld) which connect the human iso-agglutinins with properties of certain animal blood obviously offer a method for the identification of the factors A and B, independent of the frequency of these factors in human beings.[12]

SUMMARY.

In human sera there exist commonly agglutinins—"cold" agglutinins—differing from the well-known iso-agglutinins in that their effect is generally much more diminished by increased temperature. These "cold" agglutinins, apparently related to and in part identical with auto-agglutinins, display a certain specificity (Bialosuknia and Hirszfeld) and under suitable conditions their effects appear as atypical (minor) iso-agglutinin reactions. The sensitiveness to changes in temperature warrants a differentiation of these substances from the common iso-agglutinins, although agglutinins exist which seem to be intermediate.

Very probably there exist other individual differences of human bloods than those disclosed by the ordinary iso-agglutination tests.

Strong anomalous iso-agglutinin reactions occur rarely. Neither these nor the minor reactions mentioned previously or the differences disclosed by the use of animal sera have been studied sufficiently to be arranged in a practicable classification.

The division of human bloods into four groups is still adequate for ordinary purposes.

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[12] The full text of a remarkable paper by Bernstein (59), in which a new theory of the iso-agglutination factors is given, appeared after the completion of the present article. Since the factors considered by him are genetic and are possibly not demonstrable serologically, his theory need not change the conception of the factors involved in the direct serological tests. The observations by Hooker and Anderson (Bernstein, l. c., p. 248) which seem to demonstrate the presence of a special factor in the blood cells of group I have reference only to the blood of one individual, and do not necessarily have general application.

The iso-agglutinins acting on corpuscles of group II can be separated into two qualitatively different fractions which may be designated as α and α^1.

Among the corpuscles of group II (and IV) there are two varieties distinguishable by the different susceptibility to the agglutinin α^1.

Some sera of group IV contain agglutinins of the type α^1 effective on certain corpuscles of group II.

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STUDIES ON X-RAY EFFECTS.

XIV. The Effect of X-Ray on the Division Rate of Paramecium.

By ROBERT T. HANCE,* Ph.D., and HARRY CLARK, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Reports on the biological action of x-ray have been contradictory owing largely to the fact that, in the absence of a unit of dosage, accurate comparison of results has been impossible. Furthermore, it has generally been considered that dosage could be disregarded as it was to be expected that larger doses would have simply a more pronounced effect than small ones. As a matter of fact, this idea is erroneous for it has been shown that doses of different intensity may have diametrically opposite effects on a tissue (Murphy and coworkers).

With the development of an x-ray apparatus capable of delivering rays of a constant quality and intensity (Clark), a major difficulty has been eliminated. For the purposes of the present work, paramecium was selected as the biological material because its rapid multiplication renders possible the numerical expression of any reaction to radiation. This selection was made in spite of the fact that no protozoon or simple metazoon has thus far been demonstrated to give any certain response to x-ray exposures. But the ease with which such forms can be handled in large numbers either in mass cultures or individually and the facility with which they may be subjected to a variety of conditions have seemed to make it worth while to investigate anew their response to radiation.

Since reproduction is one of the most easily affected vital functions, we have chosen to test the action of various doses of x-ray on the division rate of these unicellular organisms.

Material.—Two races of paramecium have been utilized; *i.e.*, *Paramecium caudatum* and *Paramecium multimicronucleatum*. For brevity, the former will hereafter be called Race C and the latter Race M. In general appearance they

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* Fellow in zoology of the National Research Council.

are similar although Race M is the larger. Race C has but one micronucleus whereas Race M has four; and Race C has two contractile vacuoles while Race M has from two to seven, depending on environmental conditions (Hance, 1917). Although the paramecia used in the experiments came originally from two pure lines, in many of the experiments the additional precaution was taken of starting new lines from single individuals in order to insure a uniform physiological make-up.

About 15,000 animals were observed individually and many times that number were studied in mass cultures.

Method.—A standard hay infusion was made daily, with 0.5 gm. of hay in 100 cc. of spring water. The mixture was boiled for 5 minutes. For containers, Syracuse watch-glasses boiled in water containing a trace of paraffin were used for the small mass cultures or the isolated individuals. The thin film of paraffin on the glass resulting from this treatment prevented the medium from adhering to the sides of the dishes and rendered easier a complete survey of the animals in the dish.

In order to eliminate the secondary radiation from the glass container in the preliminary experiments, the paramecia were placed in 1 or 2 inch sections of paper soda straws one end of which was plugged with paraffin. With a slender tipped pipette it was possible to place a single individual in such a tube and to recover it without difficulty after the exposure to x-ray. With one end of the straw stopped the surface tension was sufficient to hold the liquid in place during the manipulation. The radiated animals were seldom in the straws more than 1 hour and the absence of any deleterious effects arising from the sojourn therein was shown by the fact that controls allowed to remain inside for 2 days were apparently unharmed. When it became evident that no considerable differences were to be found as far as the rate of division was concerned, the tube was discarded for the simpler one of exposing the animals in the watch-glasses.

The X-Ray Outfit.—The x-ray outfit used in this work has been described (Clark). A broad focus Coolidge tube mounted in a lead-lined cabinet and kept cool by means of a fan is operated on 60 cycle current rectified by kenetrons. Ionization measurements made with apparatus previously described (Clark) have shown that the outfit may be kept in operation continuously without a variation of current or voltage exceeding 2 per cent. In the experiments the rays were produced at 30 kilovolt peak and 22 milliamperes, filtered through very thin cardboard, and used at a target distance of 25.5 cm. Under these circumstances the rays produced, according to our measurements, about 6×10^{12} pairs of ions per gm. per second in air.

EXPERIMENTS.

Experiment 1. To Test the Effect on the Division Rate of Single Exposures to X-Ray.—

(*a*) Samples of the two races of animals were placed in soda straw tubes and exposed to the standard dose used in these experiments for 1, 2, 5, 10, and 20

minutes. One-half of the controls were also kept in tubes for the duration of the exposures and the other half were kept in watch-glasses. After exposure five animals from each batch were placed in five individual watch-glasses and the division rate noted for 5 days. No difference in the division rate of the two sets of controls was observed. The samples of both races exposed for 1 and 2 minutes showed no reaction, while the division rate of those subjected to the same treatment for 5, 10, and 20 minutes was slightly lowered for 2 days, after which it slightly exceeded that of the controls. At the end of 5 days the average number of divisions per day was:

	Race C.	Race M.
Control...	0.48	0.6
X-ray (all exposures)..............................	0.46	0.6

No differences in the effects of the various lengths of exposure were noticed and the total number of divisions of both x-rayed and control lines were the same at the end of 5 days as shown above. The tests involving 10 minute exposures have been repeated three times, and the 20 minute exposures eight times always with similar results. See Charts 1 and 2, Graphs 1 and 6.

(b) In all of the following experiments mass cultures of the two races of paramecium were first exposed in watch-glasses. Then from each exposed culture from ten to twenty-five animals were isolated in individual watch-glasses and the division rate followed for from 5 to 15 days. The average rate of division was obtained by dividing the total number of animals found in all of the watch-glasses by the number of individuals originally isolated. In this series of experiments exposures lasting 1, 1½, 2, 3, 4, 5, and 6 hours were used. Exposures up to 3 and 4 hours long failed to produce any more marked or different effects on the division rate than those lasting less than 1 hour but after the 5 and 6 hour treatments the division rate was slightly increased over that of the controls. As the results seemed to be complicated with temperature the detailed account is omitted here to be reported when more complete data are available. With the exception of the 1½ hour period the other exposures have been repeated from two to eight times. See Charts 1 and 2, Graphs 2, 3, 4 and 7, 8, 9 for a diagrammatic representation of the results reported above.

As the protocols show, paramecia were exposed to a range of the x-ray for periods of from 1 minute to 6 hours. Even the largest dose proved insufficient to cause the death of the animals. It was observed, however, that there was a slight but constant depression in the division rate lasting from 2 to 4 days and occasionally for 5 days. After this period the x-rayed lines divide slightly faster than the controls. This
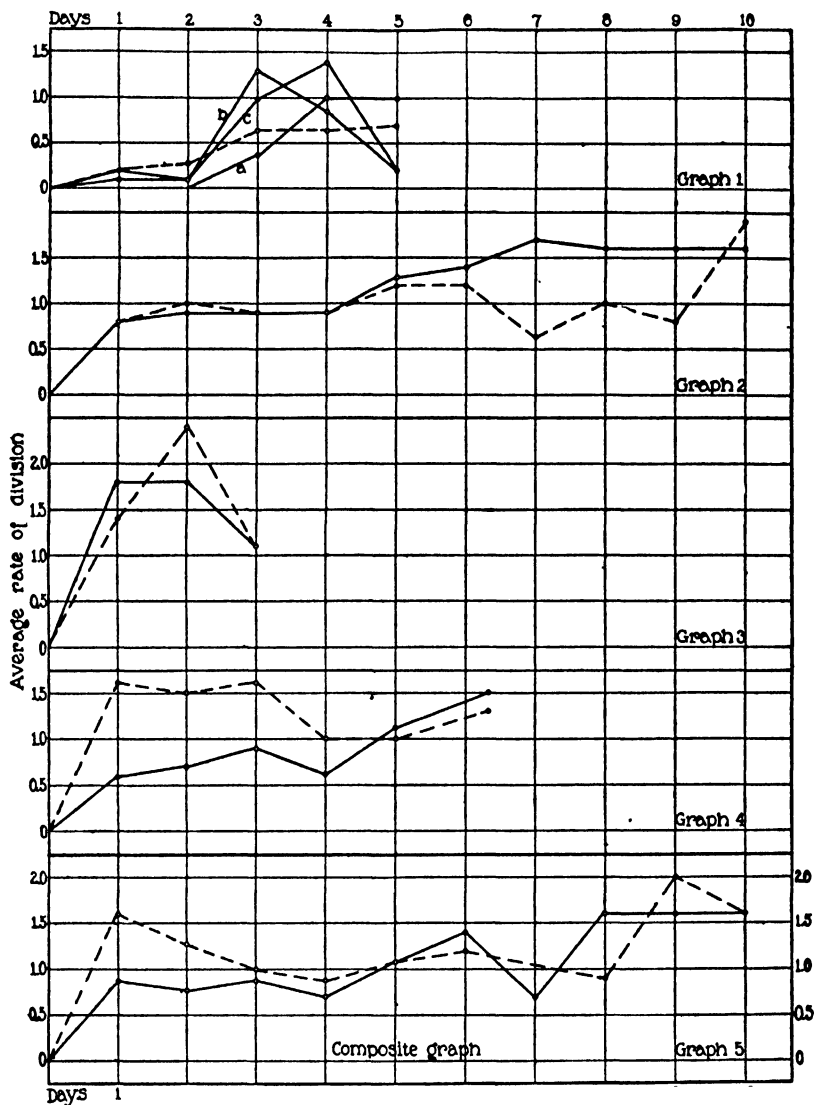
CHART 1. Graphs illustrating the effect of x-rays upon the division rate of *Paramecium caudatum*. The rates of division in tenths of a division are plotted vertically, while the number of days following exposure to x-ray are given horizontally. In all the graphs the broken lines represent the division rate of the control or non-radiated paramecium, the unbroken lines the radiated forms. Graph 1. Curve *a*, 5 minute exposure; curve *b*, 10 minute exposure; curve *c*, 20 minute exposure. Graph 2. 1 hour exposure. Graph 3. 2 hour exposure. Graph 4. 4 hour exposure. Graph 5. Composite graph made by averaging the figures upon which the first four graphs are based.

acceleration was about sufficient to offset the depression so that after awhile the x-rayed and control strains had produced about the same
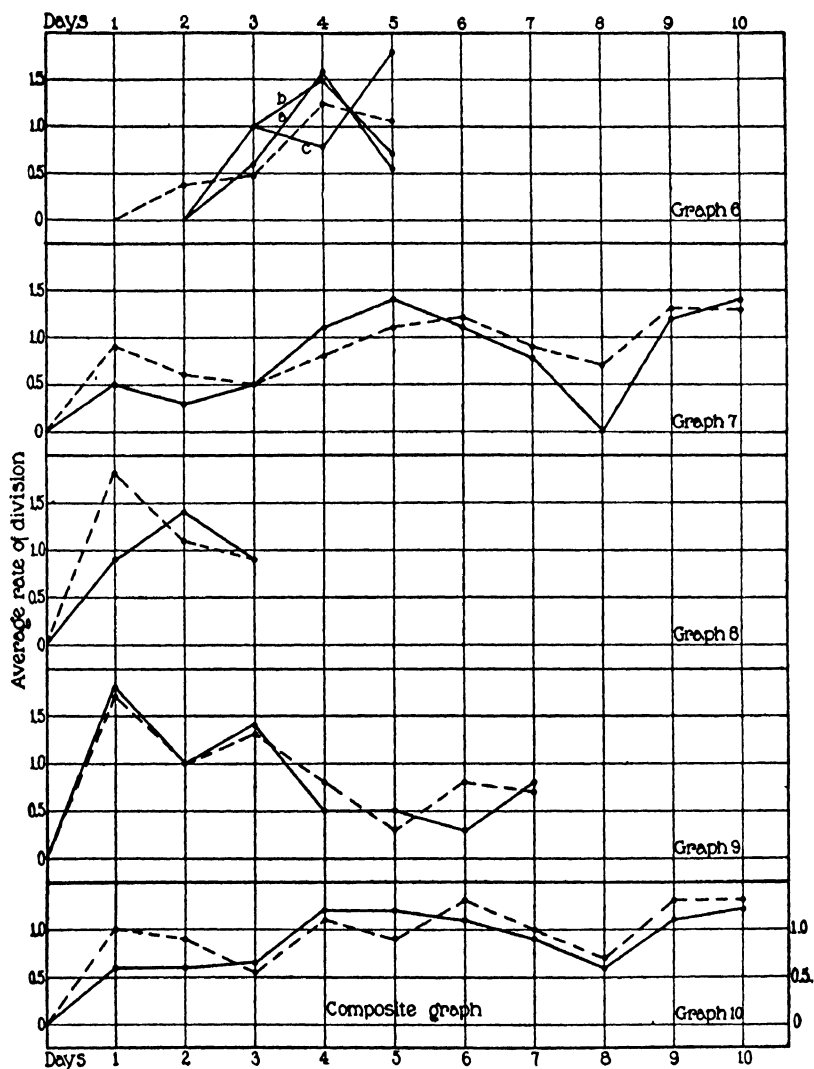
CHART 2. Similar to Chart 1 except that the reactions of *Paramecium multimicronucleatum* to x-ray are illustrated. Symbols and descriptions as in Chart 1, Graphs 6 to 10 being comparable in order to Graphs 1 to 5.

number of individuals. Both races of paramecium responded in the same manner. The period of depression was practically as marked

from the short exposures as from the longer ones although some incomplete data indicate that long exposures cause an initial stimulation rather than a depression of the division rate.

Experiment 2.   To Test the Effect on the Rate of Division of Repeated Exposures to X-Rays.—

(*a*) The exposure was repeated once within 24 hours after the first dose.

Twenty individuals of both races were isolated in separate watch-glasses and for 2 weeks their normal rate of division was recorded.   At the end of this period one animal from each line was isolated and allowed to multiply until there were four descendents.   Two of these were held as controls and the other two were placed in paper straw tubes and x-rayed for 1 hour.   The next day one of the two rayed specimens or its descendents were again exposed for 1 hour.   The rate of division was noted each day for 6 days.   In all cases, with but one exception, the animals exposed twice behaved as did those that had only one exposure.   The rate of division was temporarily lowered but returned to the normal in about 3 days.   This experiment has been repeated six times with comparable results with the single exception noted in which the forms died that were exposed twice.   In this case, of fourteen paramecia x-rayed on 2 successive days, ten died on the 1st day after the second exposure without dividing, one died without dividing on the 3rd day, one divided and died the 2nd day, one divided and died the 3rd day, and one divided after 3 days and lived.   These results have not been again obtained.

(*b*) Exposure repeated twice a day for 8 days.

Mass cultures of both races were exposed in watch-glasses for 10 minutes, at 4 p.m. and at 10 a.m. for 8 days.   The animals were exposed fifteen times in all without any obvious reduction in their number as compared with the controls.   At the end of the period all appeared slightly swollen and somewhat more sluggish than usual.

(*c*) Three exposures about 12 hours apart.

The technique was similar to that used in (*b*) except that after each exposure ten individuals were isolated from the mass culture and their division rate followed.   Two lengths of exposure were used, 15 and 30 minutes.   The first two exposures lowered the rate of division below that of the controls although after the second it was not as much lowered as after the first.   After the third exposure there was no depression, and, in the case of the 15 minute sample, there was a slight increase in the rate of division.   The average number of divisions for the first days after each of the exposures is shown below.

	Time exposed.		
	1	2	3
15 min. exposure........................	0	0.6	1.4
30 " "	0	0.4	1.2
Control.................................	0.2	0.9	1.2

At the end of 4 days the average division per day for both control and radiated animals was 1.

(d) Exposure repeated at intervals of 3 or 4 days.

Since our early work seemed to show that the division rate returned to normal in about 3 days, mass cultures were rayed every 3 or 4 days to determine whether a more enduring effect could be produced. Mass cultures of both races were placed in watch-glasses. One culture of each was held as controls and the other cultures were rayed for 20 minutes. Four individuals from each x-rayed culture were isolated and the rate of division followed for 3 and in some cases 4 days, when the culture was again exposed to x-rays for 20 minutes. This was repeated four times in all and after each exposure four paramecia were isolated from the controls and the x-rayed samples. The C race showed the usual initial depression in the rate of division followed by a complete recovery in 4 days. Each succeeding exposure produced the same effect. The M race had its division rate lowered after the first exposure but after the succeeding exposures it was slightly raised. In all cases, however, the rate of division of the x-rayed samples and of the controls was approximately the same at the end of 4 days.

In the observations described under Experiment 2, we have attempted to extend the initial depression of division by repeating the exposure once or twice daily or at intervals of 3 or 4 days. As many as fifteen exposures have failed to cause a further definite decline in the rate of multiplication or to continue the original depression. One exception to this general statement should be recorded. A number of individual paramecia were rayed for 1 hour and half of them were given a second exposure the following day. During the period that it took the controls to divide five times, the animals receiving a single x-ray dose divided twice and those that had two doses with one exception failed to divide at all and died in from 1 to 3 days. Thus far it has not been possible to duplicate this result.

Repeated doses on mass cultures of paramecium have consistently caused the animals to round out so that they appeared slightly swollen, and to become somewhat sluggish in their movements; but no lethal effect has been noted.

The data presented in Experiment 2 (c) and (d) seem to indicate that the M race may develop what might be termed an immunity to x-ray after several doses. What actually happens may be that the animal approaches the limit of its reactive power, after which it shows either less or no reaction to this physical agent. In the table of Experiment 2(c) the second dose produced less effect than the first,

and the third produced no change in one case and a slight acceleration of division in the other.   The same phenomenon is recorded in Experiment 2 (*d*).   In this instance the exposures were 3 days apart and after the first dose the division rate was initially stimulated rather than depressed.   In both cases, however, the number of animals produced at the end of a given period as in the case of the controls was the same.   The length to which this progressive inactivation, if it may so be called, may be carried has not as yet been determined.

To determine whether one end of the cell was more sensitive to x-rays than the other, the anterior and posterior ends of radiated animals were isolated after fission and followed for several generations. No differences in division behavior were noted.

To discover whether exposure to x-rays would be more potent during the process of fission (mitosis) the division of the individual was timed so that it would occur during the process of radiation.   The descendents of these animals were studied for several generations but the results were not different from those described above.

DISCUSSION.

The inability of earlier workers to obtain any results after exposing protozoa or small metazoa to x-rays is probably due to the fact that they were largely concerned with attempts to determine a lethal dose. Since the animals did not succumb even to very long exposures it was concluded that they are non-sensitive to this physical agent.   To demonstrate the reactions of paramecium to x-rays as reported above it was necessary to observe a large number of individuals and to follow their descendents for several days.

The slight initial check on cell division followed by a rapid recovery is the most obvious of our results.   The slight stimulation of the rate of division which occurred after exposures of 5 and 6 hours as well as the results (with Race M) after repeated exposures at intervals ot several days suggest that the reaction of paramecium to x-radiation may be reversible.   Treatments lasting for 10 minutes to 3 or 4 hours depress division, while longer or repeated exposures may under some conditions raise the reproductive rate.

While it is not possible on the basis of the present data to draw

conclusions regarding the character of the physiological reactions, certain inferences seem warranted. The actual mechanism of reproduction (mitosis) cannot be affected since animals radiated while dividing behaved no differently than the others. It seems more probable that the reaction to x-rays must be a more or less general one throughout the organism, since experiment has shown that one end is no more affected than the other, and since, furthermore, there is apparently a residuum of the primary effect remaining for several days, even after the animal has divided. The residual effect is indicated by the lack of a reaction or the lessened reaction to later exposures at various intervals. This residual effect disappears in 3 or 4 days as far as its influence upon cell division is concerned.

We are able to offer no direct evidence as to why protozoa or small metazoa prove so slightly reactive to doses of x-ray that are many times more than sufficient to kill a small mammal. It may be owing to the circumstance that paramecia very rapidly react to their maximum, after which no change in them occurs.

SUMMARY.

Two races of paramecium were submitted for varying lengths of time to x-radiation and a large number of individuals were observed to determine the effect on the rate of division. The division rate of both races suffered a slight initial depression lasting for 2 to 5 days following the exposure. This depression is followed by complete recovery.

Within rather wide limits the length of the exposure has, in these experiments, made no appreciable difference. Apparently the maximum effect of the x-rays is produced by relatively short exposures. Continued radiation produces little further change until exposures of 3 and 4 hours are used, when precisely the opposite results are obtained from those obtained with shorter exposures.

Doses repeated at various intervals have in general failed to interfere more markedly with the division rate than a single dose. Repeated radiation causes the cells to become slightly swollen without apparent interference with their viability.

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STUDIES ON THE RELATION BETWEEN TUMOR SUSCEPTIBILITY AND HEREDITY.

III. SPONTANEOUS TUMORS OF THE LUNG IN MICE.

BY CLARA J. LYNCH, PH.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In discussing cancer from the standpoint of heredity the present tendency is to regard susceptibility to the development of tumors of each tissue or of each organ as if inherited separately. In a previous communication[1] from this laboratory evidence was presented in favor of the view that susceptibility to tumors of the mammary gland in mice is influenced by the laws of heredity and that it apparently behaves as a dominant though variable character. The present paper is concerned with the inheritance of susceptibility to tumors of the lung in mice.

Lung tumors as well as mammary gland tumors are not uncommon in mice. They have been reported by Livingood,[2] Tyzzer,[3] Jobling,[4] Haaland,[5] Slye, Holmes, and Wells,[6] and others. Tyzzer[3] states that growths appear to be more frequent in the lung than in any other organ. In 70 tumor mice which he examined, 83 neoplasms were found. 74 per cent of all the individuals had pulmonary tumors and 62 per cent of all the tumors were in the lung. A stock of mice which was under observation for 2 years yielded 12 mice (9 females and 3 males) with lung tumor from 500 autopsies. Haaland[5] found pulmonary tumors next in frequency to mammary tumors. Complete statistics are not given. In the

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[1] Lynch, C. J., *J. Exp. Med.*, 1924, xxxix, 481.

[2] Livingood, L. E., *Bull. Johns Hopkins Hosp.*, 1896, vii, 177.

[3] Tyzzer, E. E., *J. Med. Research*, 1907, xvii, 155, 199; 1909, xxi, 479.

[4] Jobling, J. W., Spontaneous tumors of the mouse, Monograph of The Rockefeller Institute for Medical Research, No. 1, New York, 1910, 81.

[5] Haaland, M., *Ann. Inst. Pasteur*, 1905, xix, 165; *4th Scient. Rep. Imperial Cancer Research Fund*, London, 1911, 1.

[6] Slye, M., Holmes, H. F., and Wells, H. G., *J. Med. Research*, 1914, xxx, 417.

Slye stock[6] also lung tumors were found to be second to mammary gland tumors in frequency.   160 mice with pulmonary nodules were observed in 6000 autopsies (2.7 per cent) and one-third of all tumors found arose in the lung.   A large number of cases have been discovered in this laboratory.   As a result of the practice of making autopsies on all mice data have accumulated regarding the occurrence of pulmonary tumors in mixed populations and also in certain strains of mice that have been closely inbred.   Since in many of the groups under observation a large number of the individuals of the more recent generations are not yet dead this preliminary report covers results from only a few of the earlier crosses in which the data are practically complete.

Several investigators have already published contributions to the subject of the hereditary aspect of tumors of the lung.   Tyzzer[3] observed the tumor incidence in several inbred families of mice.   In the largest group (Family A) there were 98 descendants belonging to several generations derived from an original mating of a mouse which never developed tumor with a female which was found to have a cystadenoma of the lung.   Of 62 which died after reaching full maturity 20 developed one or more tumors, 17 being pulmonary tumors.   If the mice of this family are classified according to whether they are the offspring of parents without tumors or of parents one of which developed a tumor, it is found that in the first group, 9 mice in a total of 67 (13 per cent) were tumor mice while in the second group 11 out of 28 (39 per cent) developed tumors.   If only those individuals which lived more than 6 months are included in the estimates, among the 40 offspring born of tumor-free parents 23 per cent developed tumors while among 24 mice with one tumor parent 50 per cent developed tumors.   Tyzzer concluded that although the numbers dealt with were small, the data tended to indicate that there is a stronger tendency to the development of tumors in the offspring of parents with tumors than in the offspring of parents which are free from tumors.

Slye, Holmes, and Wells[6] have published data from 6000 autopsies.   In their stocks lung tumors do not ordinarily appear in mice younger than 12 months and about one-third of the individuals died under that age.   155 mice with lung tumors were investigated from the standpoint of heredity.   Of these 146 were found to have a tumor ancestry but 9 of the cases of lung tumor appeared in mice without tumor ancestry and these 9 tumors were all benign.   Since one-third of the mice autopsied were from non-cancerous strains, if inheritance played no part, there should have been 50 cases instead of 9 in these strains.   The authors cited believe that in mice without cancer ancestry inflammatory reactions in the lungs seldom become neoplastic in character; that if excessive proliferation occurs in such individuals it does not exceed that of a slight papillary growth; and that heredity modifies the nature or degree of reaction to injury.

In the present paper no attempt has been made to classify the tumors according to the degree of malignancy shown by them.   The histological aspect of tumors of the lung in mice was first described by

Livingood[2] and it has been the subject of so many communications since then that a detailed account is unnecessary here. A considerable variation exists in their behavior. Many show no evidences of malignancy while others appear to be actively growing, infiltrating the surrounding tissues in the lung or the mediastinum. They are often multiple. The neoplastic epithelium frequently resembles the bronchial epithelium, although examples in which it is like thickened alveolar epithelium are also found. We have encountered no tumors in this material like the epidermoid carcinoma of the lung described by Tyzzer. It is sometimes difficult to distinguish between neoplastic and inflammatory hyperplasia and several cases in our material have been classed as doubtful. Under this heading are also included a few mice in which pulmonary nodules were found at autopsy but in which postmortem changes made a histological verification impossible. The growths vary greatly in size. Sometimes they are so small as to escape detection at autopsy and are found only by chance in sections. Serial sections of the lung were not made so that in general only those nodules large enough to be visible to the naked eye are included in the data. The mice were allowed to live under conditions as nearly natural as a laboratory permits and the tumors under discussion were "spontaneous." Besides the lung tumors there were found in the material 7 sarcomas, 3 of which were in animals which also had lung tumors, and 8 epitheliomas of the jaw, 3 of which coexisted with tumors of the lung. One mouse with a lung tumor had a carcinoma in the ovary and 2 mice had carcinomas of the stomach. There were also a large number of tumors of the mammary gland. In fact the stocks of mice studied served as a source of supply for mammary neoplasms. The majority of the mice developing them were used in other experiments or disposed of outside this laboratory and so few of the records dealing with such animals, valid for the condition of the lung, are available that it was decided to omit them in the interest of a more uniform basis for conclusions. There are very few data from other laboratories regarding the percentage of lung tumors which occurred in the total number of mice with mammary gland tumors and also in the total number of mice free from such growths. Jobling, in a small series of mice with mammary neoplasms, found that pulmonary growths occurred in about one-third of the individuals while none appeared in several

thousand mice free from tumors of the mammary gland.   The ages of these mice are not given.   The few facts we have accumulated which bear on this point would tend to indicate that in our stock lung tumors coexist with mammary gland tumors in about the same per cent with which they occur in individuals free from mammary gland tumors.   In an inbred strain in which the rate of incidence of lung tumors was 6.7 per cent among females free from mammary gland tumors there were 23 animals developing tumors of the mammary gland, in which the lungs also were examined.   Only one of these had developed a primary pulmonary tumor.

The data which have been accumulated furnish information upon the rates of incidence of spontaneous tumors of the lung in mice (1) in relation to age and sex, (2) in two inbred strains of mice, (3) in the offspring from parents with and without pulmonary tumors, and (4) in the offspring from crosses between strains that have a high and a low rate, respectively, in regard to tumors in the lung.

1. Rates of Incidence of Spontaneous Tumors of the Lung in Relation to Age and Sex.—It has been noted that tumor rates vary markedly with the age of the individuals concerned although it is not clear whether this is a direct effect of the "age" of the tissues or indirectly due to the fact that a long life offers more opportunities for exposure to the various forms of chronic irritation which induce neoplastic disease.   The earliest age at which a lung tumor has been found in these strains is 8 months and the greatest age is 40 months.   They occur most frequently in mice of about 24 months or older.   In one group of 1500 autopsies upon mice from the general stock the maximum rate of lung tumor incidence was 45 per cent at 26 months of age.   In another group of about equal size the maximum rate of 38 per cent was reached at 29 months.   The two groups taken together give a lung tumor incidence of about 17 per cent in 2300 mice which were over 7 months of age and 11 per cent in 3500 mice of all ages.   These figures do not include mice with mammary tumors.   We can follow the monthly fluctuation of the tumor rates in these two groups combined, in Text-fig. 1.   This shows the actual numbers of mice dying at each age and the percentage of tumor mice occurring among them.   At the younger ages (8 to 12 months) the tumor rate lies at the low level of 1 to 3 per cent.   Beginning with the 13th month there is a gradual

increase in the rate and from the 22nd through the 29th month it is maintained at a level of about 30 per cent. At 26 months a rate of 39 per cent is shown and at 29 months it is almost as high. After the 29th month the rate declines slightly. The large percentages shown at 33 months and older are based on less than 10 mice in each group. If these individuals are classed together the incidence for the total number is 38 per cent, about the same rate as that given at 26 months. It is interesting to note that 2 mice died at 39 months and 2 at 40 months, and in each case 1 of the individuals was free from growths in the lung.

----- Actual No. of mice dying at each age
——— Per cent of mice with lung tumor
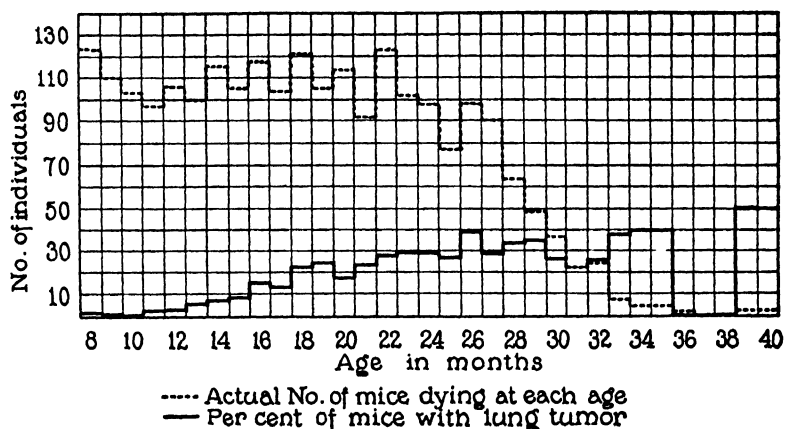
TEXT-FIG. 1.

Sex is probably not of importance in determining the occurrence of primary tumors of the lung. Slye, Holmes, and Wells report that 57.4 per cent of the lung tumors in their stock were found in females and 42.6 per cent in males. The reverse condition was found to exist in our stocks, as the higher percentage was shown by the males. Reference to Text-fig. 6 which gives ratios for the males and females of mixed ancestry shows that the percentage of lung tumors at the different age periods was consistently higher for the males than for the females. For the males of all ages combined it was 50 per cent compared with 26 per cent for the females. In Text-fig. 3 we see that also in the inbred Strain 1194 the males have a higher lung tumor rate (13 per cent) than do the females (4 per cent) and in the inbred Bagg strain 35 per

cent of the females had lung tumors whereas 45 per cent of the males developed them.   In the larger group of 2300 individuals from a variety of sources the rate of incidence was 16 per cent for the females and somewhat greater, 22 per cent, for the males.   Whatever factors may explain the high per cent of tumors among the males in certain instances in our stock (it was frequently 2 or more times as high as among the females) sex certainly is not nearly so potent a factor in determining the occurrence of tumors in the lung as it is in affecting tumors of the mammary gland, which are restricted almost entirely to the female sex, having been rarely reported in males.

2. *Rates of Incidence of Spontaneous Tumors of the Lung in Two Inbred Strains of Mice.*—Different strains of mice show differing rates of incidence of tumors of the lung but two inbred strains have been discovered which present a marked contrast to each other.

Strain 1194 is one of the Lathrop stocks and was derived originally from a cross between a male of one tumor strain and 2 females from another strain. Their descendants were inbred probably rather closely for four generations before they were acquired by this laboratory.   One pair of mice in the fifth generation gave rise to the present strain and their offspring have been bred brother by sister (or rarely, cousin by cousin) since that time.   These data include individuals from the sixth to the fifteenth generation.   Most of the mice are black agouti like the wild house mouse though a few are pink-eyed or brown agouti.   The stock has a low rate of incidence for tumors of the lung but a high rate for tumors of the mammary gland.   Growths may appear in the mammary gland when the mouse is but 5 months of age but in this strain they usually appear at about 12 months.   65 per cent of the females that have been bred and live to be 6 months old and 20 per cent of those that have not been bred have mammary gland tumors Tumors of the lung are noted only at autopsy.   Though they may appear when the mouse is but 8 months old in the general stock, in this strain the youngest age at which a lung tumor was found was 18 months.   In all, there were but 14 animals with lung tumor out of a total of 208 mice that lived to be a year old— a rate of 6.7 per cent.

For comparison with Strain 1194 we may cite the Bagg strain of albinos This stock in contrast with Strain 1194 has a high incidence of lung tumors and a lower incidence of mammary gland tumors.   It is impossible to say whether or not any significance attaches to the fact that each strain gives a high and a low tumor rate but in the opposite type of tumor.   The data cover a group of 30 or 40 mice from a stock which had been rather closely inbred for several years previous to their acquisition by us and also two generations from a half dozen pairs which were inbred brother by sister.   Among females which have been bred,

mammary gland tumors occur in about 27 per cent and in 4 per cent of females over 6 months that have not been bred; while in a total of 135 individuals over 12 months tumors of the lung are found in 37.04 per cent.

Since age and sex in regard to tumor production are of interest, the distribution of the individuals in the two strains is presented graphically in Text-fig. 2 and a summary of the ratios arranged in age periods of 6 months each is given in Text-fig. 3. The mortality of females under
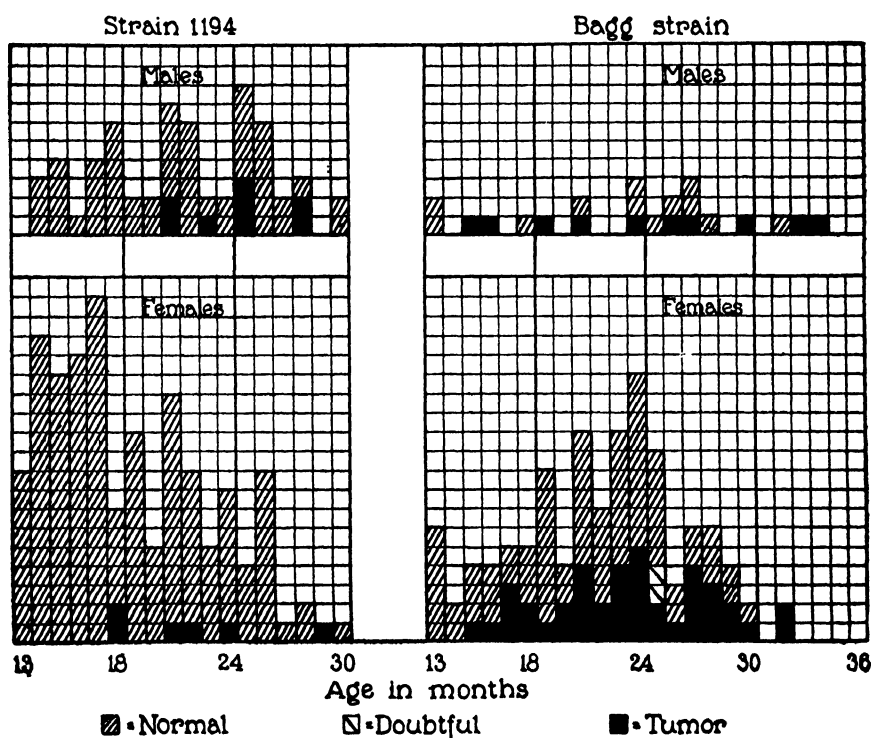
TEXT-FIG. 2.

18 months of age was greater in Strain 1194 than in the Bagg strain and tumors did not appear until the 18th month in the first strain as compared with the 15th month in the Bagg albinos. Although some of the totals are small, in each of the age periods for both males and females the ratio of tumor to non-tumor individuals is higher in the Bagg strain than it is in Strain 1194. Among the Bagg males of all ages over 12 months there were 10 mice with lung tumors and 12 non-

tumor mice (45 per cent of lung tumors), whereas in Strain 1194 there were 8 males with tumors and 52 non-tumor males (13 per cent). Among the females, the ratio in the Bagg strain was 40 tumor mice to 73 non-tumor mice (35 per cent) and in Strain 1194 there were 6 tumor mice to 142 non-tumor mice (4 per cent). Combining individuals of both sexes and all ages the rate of incidence of tumors of the lung in the Bagg strain was 37.04 per cent ± 2.80 in a total of 135 mice and in Strain 1194 it was only 6.73 per cent ± 1.17 in a total of 208 mice. The difference between the tumor rates of the two strains is 30.31 ± 3.04. Since the difference is almost 10 times the probable error the

Females

Strain	Age, 13-18 mos.		19-24 mos.		25-36 mos.		Total		
	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	Per cent
Bagg strain	19	7	38	18	16	15	73	40	35
Strain 1194	77	2	48	3	17	1	142	6	4

Males

Strain	Age, 13-18 mos.		19-24 mos.		25-36 mos.		Total		
	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	Per cent
Bagg strain	3	2	3	3	6	5	12	10	45
Strain 1194	18	0	18	3	16	5	52	8	13

TEXT-FIG. 3.

chances are enormously against its being due to fluctuations in sampling. We may conclude that it is undoubtedly significant.[7]

3. *Comparison of Rates of Incidence among the Offspring of Parents Which Have and of Parents Which Have Not Developed Primary Tumors of the Lung.*—Records are available of a very mixed population derived from crosses between mice from a variety of sources. The offspring from these matings may be grouped in three classes: mice (1) from parents in which no lung tumors were found, (2) from parents one of which developed a lung tumor, and (3) from parents both of which

[7] The probable error of the percentages was calculated by the method given by Haldane (Haldane, J. B. S., *J. Genetics*, 1919, viii, 291).

developed lung tumors. In certain cases the autopsy record of one of the parents was not available for reasons already stated. The offspring of such a parent were included in the data, the parent being classed as a non-tumor mouse. This procedure is justified because the
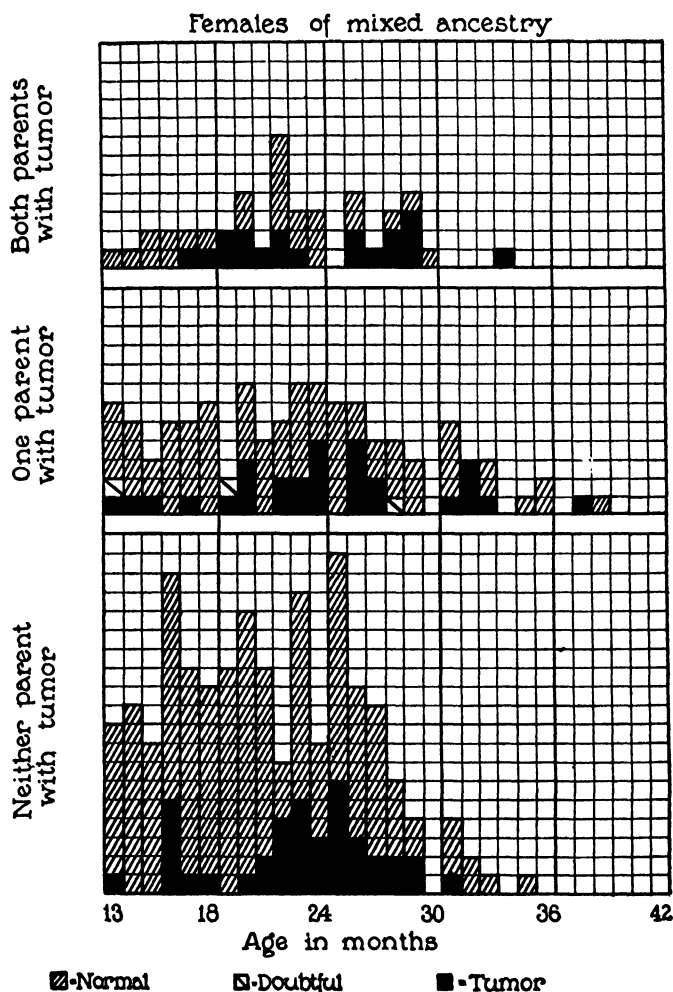
TEXT-FIG. 4.

data include also mice from parents which died younger than the earliest age at which tumors have ever been seen and because at best the classification is inexact. Probably many mice never produce tumors

although they have an inherited capacity to do so. Such individuals must be classed as non-tumor mice whereas genetically they are tumor mice. Class 1, above, which comprises mice from non-tumor parents probably includes individuals which should go in Class 2 or

Males of mixed ancestry

Both parents with tumor

One parent with tumor

Neither parent with tumor

13   18   24   30   36
Age in months

▨ • Normal   ◩ ·Doubtful   ■·Tumor
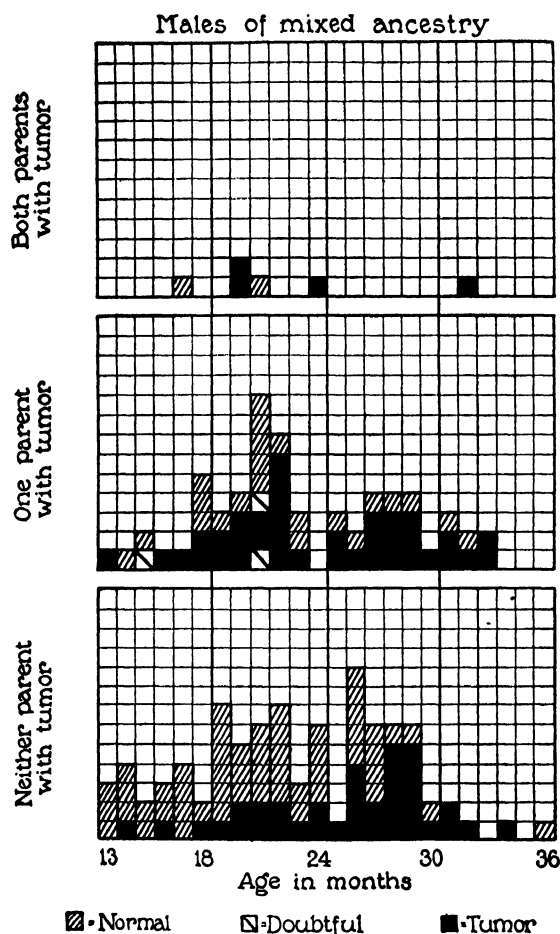
TEXT-FIG. 5

3, and Class 2 probably includes mice of which both parents were genetically speaking tumor mice and should have been put in Class 3. The males and females are grouped separately and the age distribution of the three classes for each sex is shown in Text-figs. 4 and 5. The distribution of deaths covers about the same range for the three

Females of mixed ancestry

Type of mating	Age, 13-18 mos.			19-24 mos.			25-30 mos.			31-36 mos.			37-40 mos.			Total		
	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors
0×0	59	8	12	55	15	21	34	15	31	7	1	13	0	0	0	155	39	20
T×0	25	4	14	19	12	39	16	6	27	9	5	36	1	1	50	70	28	29
T×T	8	2	20	12	8	40	5	8	62	0	1	100	0	0	0	25	19	43

Males of mixed ancestry

Type of mating	Age, 13-18 mos.			19-24 mos.			25-30 mos.			31-36 mos.			37-40 mos.			Total		
	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors	No tumor	Tumor	Per cent tumors
0×0	15	3	17.	24	10	29	12	18	60	1	4	80	0	0	0	52	35	40
T×0	5	5	50	10	14	58	5	13	72	2	5	71	0	0	0	22	37	63
T×T	1	0	0	1	3	75	0	0	0	0	1	100	0	0	0	2	4	67

0×0 = neither parent with tumor
T×0 = one parent with tumor
T×T = both parents with tumor

TEXT-FIG. 6.

classes when their respective age periods are compared.   The occurrence of the doubtful cases is indicated on the graphs but they are omitted from the summary of the ratios which is given in Text-fig. 6.

The data for both sexes show that the rate of incidence of tumors of the lung is higher among the offspring when the parents are known to have developed lung tumors than when the parents were tumor-free.

Among the females of all ages in the first class (from tumor-free parents) there were 155 non-tumor mice to 39 tumor mice, or 20 per cent; in the second class (in which one of the parents developed a lung tumor) there were 70 non-tumor to 28 tumor mice, or 29 per cent; in the third class (in which both parents had tumors) the percentage of tumors increased still more as there were 25 non-tumor and 19 tumor mice, or 43 per cent.   Among the males, in the first class there were 52 non-tumor and 35 tumor mice, or 40 per cent and in the second class, 22 non-tumor mice to 37 with tumors, or 63 per cent.   There were but 6 individuals in the third class but 4 of them developed tumors. Examining the ratios for the different age periods we find evidence of the same increase in tumor rate in the groups which have a tumor ancestry over groups with a non-tumor parentage.   Among the females between 12 and 18 months of age the rate increases slightly from 12 per cent in the first class to 14 per cent in the second.   In the third class there are but 10 mice but 2 of them (20 per cent) had lung tumors.   Females between 19 and 24 months in the first class had a tumor rate of 21 per cent; in the second class 39 per cent and in the third class 40 per cent.   In the age period between 25 and 30 months is found one of the two instances in which the tumor rate for mice in the second class fell below that of the first class.   From parents free from lung tumors there were 34 non-tumor and 15 lung tumor mice, or 31 per cent.   The number of offspring in cases in which one of the parents had a lung tumor was small.   There were 16 mice without lung tumors and 6 mice which developed them, or 27 per cent.   From crosses between 2 parents with lung tumor there were fewer individuals (5) without lung tumor than there were with lung tumor (8).   The numbers in the age period from 31 to 36 months are small but the percentage of tumors increases from the first to the third class.

Among the males, the numbers in the several age periods are small

but the percentages are consistently higher in the second and third classes except in the age period of 31 to 36 months. The largest groups are found between 19 and 24 months of age. From parents free from tumor (Class 1) there were 24 non-tumor to 10 tumor mice (29 per cent); from parents one of which had a tumor (Class 2) there were 10 non-tumor to 14 tumor mice (58 per cent); there were 4 mice from parents both of which had tumor and 3 out of the 4 were tumor mice.

The two inbred strains—the Bagg albinos and Strain 1194—have not been included in the above figures. The distribution of the indi-

Females of Strain 1194

Type of mating	Age, 13-18 mos.		19-24 mos.		25-30 mos.		Total	
	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor
0 × 0	68	2	40	1	15	1	123	4
T × 0	6	0	6	1	0	0	12	1

Males of Strain 1194

Type of mating	Age, 13-18 mos.		19-24 mos.		25-30 mos.		Total	
	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor
0 × 0	13	0	18	3	16	4	47	7
T × 0	5	0	0	0	0	0	5	0

0 × 0 total for males and females - 170 no tumor to 11 tumor
T × 0 " " " " " - 17 " " - 1 "

0 × 0 - neither parent with tumor
T × 0 - one " " " "

TEXT-FIG. 7.

viduals according to age, sex, and class is shown in Text-figs. 7 and 8. In Strain 1194 the majority of the matings were between parents in which no tumor was found. Of their offspring (males and females) there were 170 which did not develop lung tumors and 11 which did develop tumors, giving a tumor rate of 6 per cent. Of the 18 offspring from matings between tumor and non-tumor parents only one developed a growth in the lung. There were no matings between 2 tumor mice. In the Bagg strain, from non-tumor parents there were 2 non-tumor mice and one individual with a tumor. From crosses in which one parent had a tumor the ratio was 15 non-tumor and 15 tumor mice and

from matings in which both parents had lung tumors the ratio was 5 to 6.   In these strains little difference is seen in the ratios from the various types of crosses.

If the available data are combined it is found that among the offspring of both sexes from non-tumor parentage there was a total of 465 mice of which 86 developed lung tumors (19 per cent); from parents one of which had tumor, there were 205 mice of which 81 developed tumors (40 per cent) and from doubly cancerous parentage

Females of Bagg strain

Type of mating	Age, 13-18 mos.		19-24 mos.		25-30 mos.		Total	
	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor
O × O	0	0	0	1	0	0	0	1
T × O	2	1	6	4	3	4	11	9
T × T	0	2	0	0	5	4	5	6

Males of Bagg strain

Type of mating	Age, 13-18 mos.		19-24 mos.		25-30 mos.		Total	
	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor
O × O	0	0	1	0	1	0	2	0
T × O	0	1	1	2	3	3	4	6
T × T	0	0	0	0	0	0	0	0

```
O × O total for males and females · 2 no tumor to 1 tumor
T × O    "      "      "     "      "   ·15 "  " · 15 "
T × T ·    "      "      "   "      "   ·5 "  " · 6 "

O × O · neither parent with tumor
T × O · one parent with tumor
T × T · both parents with tumor
```

TEXT-FIG. 8.

there were 61 progeny of which 29 had tumors (48 per cent).   These totals and the figures given in the separate analyses show that mice are more likely to have lung tumors when one of their parents had a lung tumor than if neither parent did and this likelihood is increased still further when both parents have tumors.

4. *Crosses between Strains Having a High and a Low Incidence of Tumors of the Lung.*—There were a few matings between males from the Bagg strain which has a lung tumor incidence of 37.0 per cent and females from Strain 1194 in which the lung tumor incidence is 6.7

per cent. Only one of the 2 males used developed a tumor. None of the females had growths in the lung. Of the 14 members of the first filial generation which lived to be at least 16 months old, 7 sons were free from lung tumors while 5 developed them. Neither of the 2 daughters had lung tumor though one lived to be 16 and the other 18 months old. These individuals and several more which died before tumor age were inbred to produce the second filial generation. The males of this second generation were used in another experiment since their value for the lung tumor record was at that time unknown. Of the females 49 lived to be more than 12 months old and of them 11 (22 per cent) developed lung tumors. The age distributions of the
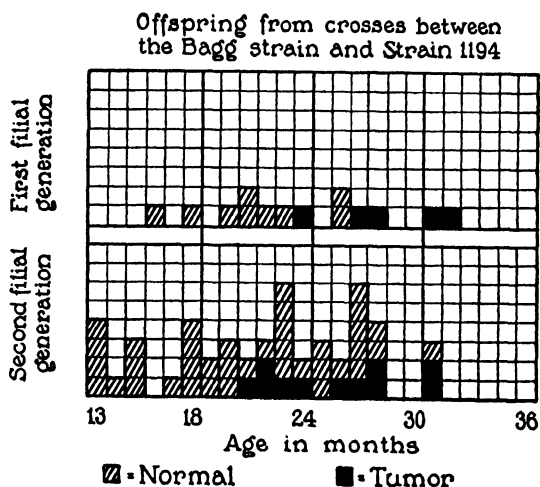
TEXT-FIG. 9.

first and second filial generations are represented in Text-fig. 9. In the first filial generation most of the non-tumor mice were younger than the individuals with tumor. The appearance of tumors in the first generation when tumor-free individuals from a low tumor stock were crossed with mice from a high tumor stock suggests that tumor susceptibility is dominant.

SUMMARY AND DISCUSSION.

1. The occurrence of tumors in the lung in mice is dependent to a certain extent upon the age of the individual. No tumors were found

in the lungs of mice less than 8 months old. They occurred with greatest frequency in mice of about 24 months or older. Mice may live to be more than 3 years old without developing growths in the lung. These facts show that the development of tumors of the lung, if hereditary, is a variable character. An individual, genetically a tumor mouse, may live to a great age without showing a tumor if the requisite environmental stimulus (external or internal) is lacking.

Sex if effective at all has a comparatively slight influence upon the incidence of lung tumors.

2. Two strains of mice were studied which exhibit differences in their rates of incidence of lung tumors that are large enough to be significant. The conflict in the evidence from different laboratories as to whether or not tumors of the lung are the commonest type found in mice, is probably to be explained on the basis of a differing hereditary tendency to such growths in differing stocks.

3. Data from a number of sources indicate that offspring from parents free from lung tumors have a lower rate of lung tumor incidence than offspring from parents one of which had a tumor. If both parents had lung tumors the rate of tumor incidence among their offspring is increased still further.

4. In crosses between mice from strains which have high and low rates of incidence of lung tumor, tumors appeared in about half of the individuals of the first generation and in about one-quarter of the second generation. If the character responsible for the development of the growths is recessive it should not be found in the first filial generation unless both parents are carrying it. There is no proof that the female parents did not carry it but since they were taken from a strain in which the incidence of the growths was but 6.7 per cent the chances seem good that they were free from it. This suggests that the character determining the incidence of pulmonary tumors may be a dominant one.

A dominant character is not expected to appear among the offspring from parents neither of which has shown the character. The numerous instances which have been tabulated in this paper of mice with lung tumors among the offspring of parents free from lung tumors, must be explained on the assumption that tumor susceptibility is not

only dominant but variable and that some of the parents which did not actually develop tumors were genetically tumor mice and had the capacity for developing tumors although it was not brought out. As we have already concluded on the basis of the relationship between age and tumor incidence that susceptibility to the development of lung tumors is a variable character our explanation of the occurrence of tumor mice derived from tumor-free parents is justifiable.

The existence of strains of mice with rates of incidence of lung tumors that differ as widely as do the two that we have studied, the relatively high incidence of pulmonary growths among mice of tumor parentage as compared with mice from non-tumor parents, and the fact that females from a strain in which pulmonary tumors are rare when crossed with individuals from a strain in which they are frequent give a fairly high rate of incidence of the growths among the first and second filial generations,—all these facts indicate that susceptibility to the development of tumors in the lung is an inherited character.

THE KJELDAHL-PREGL METHOD APPLIED TO NITRO COMPOUNDS.

BY ADALBERT ELEK AND HARRY SOBOTKA.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Kjeldahl[1] found that it was possible to convert 60 to 80% of the nitrogen of potassium nitrate into ammonium sulfate by the digestion with sulfuric acid if three parts of sugar were added to one part of the nitrate. Von Asboth[2] obtained quantitative results by this method on nitro and cyano compounds. For nitrates, however, the sugar had to be replaced by benzoic acid. For the analysis of nitro, azo and similar compounds by digestion with sulfuric acid, others suggested the addition of phenol and zinc dust,[3] phenol, resorcinol or phloroglucinol alone,[4] zinc alone,[5] potassium persulfate and zinc dust,[6] stannous chloride[7] and sulfur.[8]

The use of sugar suggested by von Asboth is not to be found in most analytical handbooks and this method has apparently fallen into disuse. In the analysis of nitro-uracil xyloside[9] we noticed that the sugar component of this compound is almost sufficient to reduce the nitrogen of the nitro group. We never had obtained satisfactory results by the various methods mentioned above, which involve a tedious additional operation and require up to six hours. We, therefore, tried the addition of glucose in the analysis of various substances

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[1] Kjeldahl, *Compt. rend. Lab. Carlsberg*, **2**, 1, 12 (1883); *Z. anal. Chem.*, **22**, 366, 381 (1883).

[2] Von Asboth, *Chem. Centr.*, [3] **17**, 161 (1886).

[3] Jodlbauer, *Chem. Centr.*, [3] **17**, 433 (1886).

[4] Margosches and Scheinost, *Ber.*, **58**, 1850, 1857 (1925).

[5] Dafert, *Z. anal. Chem.*, **27**, 222 (1888).

[6] Milbauer, *ibid.*, **42**, 725 (1903).

[7] Krüger, *Ber.*, **27**, 1633 (1894). Flamand and Prager, *Ber.*, **38**, 559 (1905).

[8] Eckert, *Monatsh.*, **34**, 1964 (1913).

[9] Levene and Sobotka, *J. Biol. Chem.*, **65**, 469 (1925).

which otherwise would have had to be analyzed by the Dumas method. The accuracy and range of the method is readily seen from the results given in Table I, where aliphatic and heterocyclic as well as isocyclic nitro compounds were tested. One g. of glucose when added to 0.1 g. of sample was found to be sufficient; digestion and distillation may be carried out in the usual manner.

TABLE I.

Results with Typical Nitro Compounds, Using Glucose.

Substance	Formula	Wt., g.	0.1 N acid, cc.	Per cent of nitrogen		Glucose, g.
				Calcd.	Found	
1. Nitrobenzoyl chloride....	$C_7H_4O_3ClN$	0.1000	5.40	7.70	7.56	1
2. β-Nitrosonaphthol.......	$C_{10}H_7O_3N$	.0960	5.50	8.09	8.00	1
3. Nitropropanol (liq.).....	$C_3H_7O_3N$	.1116	10.50	13.33	13.26	1
4. Nitroquinoline..........	$C_9H_6O_2N_2$	.0920	10.40	16.02	15.82	1
5. Picric acid..............	$C_6H_3O_7N_3$	.0924	12.10	18.37	18.32	2
		.0924	12.05		18.25	1
6. m-Nitro-aniline.........	$C_6H_6O_2N_2$	.0993	14.35	20.21	20.23	1
7. Nitro-uracil.............	$C_4H_3O_4N_3$	.1000	19.15	26.75	26.81	1
		.1000	19.10		26.74	2

On account of the great advantage offered by Pregl's[10] micro methods as to economy of both time and material, we developed von Asboth's modification of the Kjeldahl method for micro-analysis; 3–10 mg. of the sample was weighed in a small weighing tube and dropped into the flask. After addition of 50–100 mg. of glucose, 1 g. of potassium sulfate and a small crystal of copper sulfate, 3 cc. of sulfuric acid was added and digestion and distillation were carried out according to Pregl. In order to make certain that a sufficient amount of alkali had been added, a few drops of alizarin sulfonate were used as an indicator. The digestion did not involve a loss of time as compared with the usual Pregl method. The addition of a few drops of alcohol after decolorization seemed to be unnecessary with our modification. The digestion was complete after 40 minutes. Therefore, a little more than one hour was required for one estimation.

[10] Pregl, "Quantitative Organic Microanalysis," Trans. by E. Fyleman, Blakeston and Co., Philadelphia, 1925.

TABLE II.

Results in Micro-Analyses.

Substance	Wt., mg.	$N/70$ acid, cc.	Per cent of nitrogen	
			Calcd.	Found
1. Nitrobenzoyl chloride...................	11.274	4.31	7.70	7.64
2. β-Nitrosonaphthol.....................	8.238	3.37	8.09	8.18
3. Nitropropanol.......................	9.152	6.20	13.33	13.49
4. Picric acid............................	3.280	3.02	18.37	18.41
5. *m*-Nitro-aniline......................	5.460	5.58	20.21	20.44
6. Nitro-uracil..........................	4.474	5.96	26.75	26.65

Excellent checks were obtained on dried samples, although in numbers 1, 2 and 4 of Table II, samples were taken from stock bottles containing substances not labelled C.P. This fact might help to overcome the objection of industrial chemists to micro-analysis on account of the alleged danger in taking minute samples.

SUMMARY.

A micro Kjeldahl method is described which gives satisfactory results for the determination of nitrogen in nitro, azo and other similar compounds.

ON THE NITROGENOUS COMPONENTS OF YEAST NUCLEIC ACID.

BY P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

(Received for publication, November 7, 1925.)

It is firmly established that complete hydrolysis of yeast nucleic acids yields four nitrogenous derivatives. On partial hydrolysis, depending upon the temperature and time of hydrolysis, either four nucleosides or four nucleotides are obtained. On the basis of these findings the tetranucleotide theory of the structure of nucleic acid was formulated. From time to time, however, doubts were expressed as to the primary origin of the uracil compound. For a long time Steudel refuted the tetranucleotide structure, but in a more recent publication, in cooperation with Izumi,[1] he reported analytical data which, in his opinion, agreed better with the tetra than with the trinucleotide theory.

Very recently, Walter Jones, in cooperation with M. E. Perkins,[2] hydrolyzed yeast nucleic acid in a solution containing an excess of 1 per cent of sodium hydroxide at the temperature of boiling water. Under such conditions, they failed to isolate uridinephosphoric acid from the product of hydrolysis. This result led them to abandon the view of the tetranucleotide structure of yeast nucleic acid. Uridine-phosphoric acid was isolated when the nucleic acid was dissolved in a 5 per cent solution of ammonia water and the solution heated for 2 hours at 100°C. In the opinion of W. Jones these conditions suffice to hydrolyze off the amino group from the cytosine complex of the nucleic acid.

Theoretically, it is not easy to conceive that under such mild conditions as hydrolysis with very dilute ammonia, the amino group could be removed from the cytosine complex. There is also another argu-

[1] Steudel, H., and Izumi, S., *Z. physiol. Chem.*, 1923, cxxxi, 159.

[2] Jones, W., and Perkins, M. E., *J. Biol. Chem.*, 1924–25, lxii, 557.

ment which speaks against the view of Jones. Animal nucleic acid has a structure very closely resembling that of plant nucleic acid. Among its nitrogenous constituents, one is cytosine. When the two acids are hydrolyzed under identical conditions, the former yields only an insignificant quantity of uracil, whereas the yeast nucleic acid yields equimolecular proportions of cytosine and uracil and each one of these bases is obtained in equimolecular proportions with the purine derivatives.

However, the point raised by Jones is of sufficient importance to be subjected to a further experimental test. In an easy and decisive way the theory could be tested if, instead of nucleic, cytidinephosphoric acid were used for the experiment. If the view of Jones is correct, cytidine-phosphoric acid should yield uridinephosphoric under the conditions under which nucleic acid forms the latter nucleotide.

This test has now been made and the result was negative. The specific rotation of cytidinephosphoric in 5 per cent ammonia water is $[\alpha]_D^{20} = +44.0°$, that of uridinephosphoric acid $[\alpha]_D^{20} = +14.0°$. Thus, it is easy to detect the change polarimetrically.

The original rotation of cytidinephosphoric acid remained unchanged after 2 and after 3 hours of heating in a sealed tube at 100°C. From the reaction product unchanged cytidinephosphoric acid could be crystallized with a 50 per cent yield of the original material. In a parallel experiment cytidinephosphoric acid was dissolved in a 5 per cent ammonia solution and from this unheated solution cytidinephosphoric acid was obtained in crystalline form also with a yield of 50 per cent of the original material. Thus, for the present, there seem to be no sufficient grounds to alter the older view as to the tetranucleotide structure of yeast nucleic acid.

<center>EXPERIMENTAL.</center>

The cytidinephosphoric acid used in the experiment was perhaps slightly contaminated with adenylic acid. It contained 13.5 per cent nitrogen, whereas the theory requires 13.00 per cent. The specific rotation of the substance was $[\alpha]_D^{20} = +31.0°$, whereas the pure substance has a $[\alpha]_D^{20} = +44.0°$. The specific rotation of uridinephosphoric acid in the identical solvent is $+14.0°$. 0.300 gm. cytidine-

phosphoric acid was heated in a sealed tube at 100° for 2 hours.   The rotations were:

Before heating.

$$[\alpha]_D^{20} = \frac{+1.89° \times 100}{2 \times 3} = +31.5°$$

After heating.

$$[\alpha]_D^{20} = \frac{+1.89° \times 100}{2 \times 3} = +31.5°$$

In a second experiment 0.300 gm. of the substance was dissolved as in the previous experiment.   The heating was continued 3 hours. The rotations were as follows:

Before heating.

$$[\alpha]_D^{20} = \frac{+1.87° \times 100}{2 \times 3} = \times 31.0°$$

After heating.

$$[\alpha]_D^{20} = \frac{+1.87° \times 100}{2 \times 3} = +31.0°$$

2.0 gm. of cytidinephosphoric acid were dissolved in 20.0 cc. of 5 per cent ammonia water and the solution heated at 100°C. for 2 hours. The solution was then concentrated nearly to dryness.   The residue was dissolved in water and the aqueous solution was made strongly acid by means of acetic acid.   An equal volume of methyl alcohol was added and the solution was allowed to stand overnight.   The crystalline material was filtered off and dried under diminished pressure over sulfuric acid, first at room temperature and later at 55°C. The yield was 1.0 gm. and the substance contained 13.5 per cent nitrogen.

1.0 gm. of cytidinephosphoric acid was dissolved in 10 cc. of 5 per cent ammonia water.   The solution was acidified by means of glacial acetic acid and an equal volume of methyl alcohol was added.   The following day a crystalline deposit formed.   After drying it weighed 0.5 gm.

THE CONVERSION OF OPTICALLY ACTIVE LACTIC ACID TO THE CORRESPONDING PROPYLENE GLYCOL.

BY P. A. LEVENE AND H. L. HALLER.

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York.*)

A previous communication[1] dealt with the conversion of levo-β-oxybutyric acid into levo-propylene glycol through the intermediary step of levo-1-amino-2-hydroxypropane. Independently and simultaneously, identical results were reported by P. Karrer and W. Klarer.[2] At the time of our first publication we overlooked, to our regret, an earlier publication by E. Abderhalden and E. Eichwald[3] who, starting from dextro-1-amino-2-chloropropane arrived at levo-β-hydroxybutyric acid. According to Abderhalden, dextro-propylene glycol leads to levo-β-hydroxybutyric acid. In our experiments as well as in those of Karrer and his coworker, β-hydroxybutyric acid and the propylene glycol obtained from it rotated in the same direction. The causes of the discrepancy are now under investigation.

The present communication contains a report on the conversion of levo-lactic acid into levo-propylene glycol. The propylene glycol was obtained on reduction of the ester of lactic acid by means of metallic sodium by the method of Bouveault as modified in this laboratory. Efforts at converting esters of α-hydroxy acids into the corresponding glycols according to the original method were not successful. Under the procedures followed in our laboratory the reduction in the desired direction did take place although the yield of the glycol was very small. The product analyzed correctly for propylene glycol in one instance; in another, the substance contained a higher percentage of carbon than is required by theory for the glycol. However, the glycol was readily identified in the form of its diurethane

[1] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1925, lxv, 49.

[2] Karrer, P., and Klarer, W., *Helv. Chim.*, *Acta*, 1925, viii, 393.

[3] Abderhalden, E., and Eichwald, E., *Ber. chem. Ges.*, 1918, li, 1312.

derivative. The diurethane rotated to the right, that is, in the oppo-
site direction from the glycol, which rotated to the left. We have
reason to believe that the impurity which was present in a small
amount in the glycol was optically inactive, since the higher boiling
fraction of the reduction product contained a very high percentage
of carbon (C = 64 per cent) and was optically inactive. If our
observations and those of Karrer and his coworker regarding the
direction of rotation of β-hydroxybutyric acid and of propylene glycol
are correct, then dextro-β-hydroxybutyric acid and dextro-lactic acid
are configurationally related. This conclusion would be in harmony
with the fact that both in dextro-β-hydroxybutyric acid and dextro-
lactic acid the salts have a lower dextro rotation than the correspond-
ing free acids. Further work is now in progress.

EXPERIMENTAL.

In the preliminary experiments, a lactic acid of low activity was
employed. In a 2 dm. tube the syrup (sp. gr. 1.2) had a rotation of
$-1.15°$. This syrup was diluted to six times its volume with water
and boiled under a reflux condenser for 6 hours. The solution after
cooling had the following rotation.

$$[\alpha]_D^{20} = \frac{+0.20° \times 100}{2 \times 18} = +0.55°$$

The acid was converted into the ethyl ester, the rotation of which
without solvent was $-2.53°$ (sp. gr. $= 1.035, l = 1$).

$$[\alpha]_D^{20} = -2.5°$$

The ester, reduced in the manner described below, yielded a propy-
lene glycol having the following rotation in absolute alcohol.

$$[\alpha]_D^{20} = \frac{+0.08° \times 100}{1 \times 12.2} = +0.6°$$

It had the following composition.

0.1376 gm. substance: 0.2408 gm. CO_2 and 0.1312 gm. H_2O.
$C_3H_8O_2$.   Calculated.   C 47.37, H 10.52.
       Found.   " 47.72, " 10.66.

Levo-Lactic Acid.—This acid was prepared by the procedure described by Irvine.[4] The morphine salt was twice recrystallized from 50 per cent alcohol. It was then dissolved in water and decomposed with a slight excess of ammonia. The morphine was filtered off and the filtrate converted into the calcium salt. A sample of the anhydrous salt gave the following rotation.

$$[\alpha]_D^{25} = \frac{+0.21° \times 100}{1 \times 2.7} = +7.8°$$

Dextro-Ethyl Lactate.—25 gm. of thoroughly dried calcium lactate were suspended in 150 cc. of absolute alcohol and 12 gm. of concentrated sulfuric acid slowly dropped in, the mixture being thoroughly stirred with a mechanical stirrer. The mixture was heated under a reflux condenser in an oil bath (90–100°) overnight. It was then cooled, the excess sulfuric acid neutralized with potassium carbonate, and the solution filtered from salts. After removal of the alcohol the ester was distilled under diminished pressure. It distilled at 54–56°C., p = 20 mm. The yield was 14 gm. or 52 per cent of the theory. It analyzed as follows:

0.1012 gm. substance: 0.01856 gm. CO_2 and 0.0774 gm. H_2O.
$C_5H_{10}O_3$. Calculated. C 50.85, H 8.47.
Found. " 50.01, " 8.45.

The rotation without solvent was +11.05° (sp. gr. = 1.035, $l = 1$).

$$[\alpha]_D^{22} = +10.7°$$

Levo-1, 2-Dihydroxypropane.—Ethyl lactate was reduced in 10 gm. lots with sodium and alcohol in the apparatus described by Levene and Allen.[5] 12 gm. of sodium were suspended in 100 cc. of dry toluene and the mixture was heated. When the sodium was melted the stirrer was rotated vigorously in order to form a fine emulsion. 10 gm. of the ester, dissolved in 25 cc. of alcohol, were then introduced through a dropping funnel at such a rate that there was gentle refluxing. The mixture was continuously stirred. The addition of the ester required 15 minutes. In order to complete reduction and dissolve all the

[4] Irvine, J. C., *J. Chem. Soc.*, 1906, lxxxix, 935.
[5] Levene, P. A., and Allen, C. H., *J. Biol. Chem.*, 1916, xxvii, 443.

sodium, absolute alcohol was next introduced. The solution was cooled and 15 cc. of water added. Carbon dioxide was then passed into the solution until it was neutral. The sodium carbonate which settled out was filtered off and thoroughly washed with alcohol and ether. The filtrate and washings were concentrated under diminished pressure. The glycol was obtained from the residue by distillation under diminished pressure. It distilled 60–70°C., p = 1 mm. The yield was poor. It analyzed as follows:

0.1007 gm. substance: 0.01838 gm. CO_2 and 0.0976 gm. H_2O.
$C_3H_8O_2$.   Calculated.   C 47.37, H 10.52.
Found.   " 49.77, " 10.84.

The rotation in absolute alcohol was

$$[\alpha]_D^{21} = \frac{-0.65° \times 100}{1 \times 35} = -1.85°$$

Dextro-Propylene Diphenyl Dicarbamate (Propylene Di- (Phenyl Urethane)) $CH_3CH(OCONHC_6H_5)$ $CH_2(OCONHC_6H_5)$.—This substance was obtained by heating 1.6 gm. of propylene glycol with 5 gm. of phenylisocyanate under a reflux condenser on the water bath for 2 hours. It was recrystallized three times from 70 per cent alcohol and melted at 136–140°C. It analyzed as follows: .

0.0989 gm. substance required 6.30 cc. 0.1 N HCl.
$C_{17}H_{18}O_4N_2$.   Calculated.   N = 8.92 per cent.
Found.   " = 8.91 "   "

In absolute alcohol it gave a rotation of

$$[\alpha]_D^{23} = \frac{+0.56° \times 100}{2 \times 10} = \times 2.8°$$

THE STRUCTURAL RELATIONSHIP OF THE CARDIAC POISONS.

BY WALTER A. JACOBS AND ALEXANDER HOFFMANN.

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York.*)

In former communications investigations have been described which demonstrated definitely the nature of the unsaturated linking in strophanthidin. It was found that this double bond is situated within the lactone ring in all probability between the β and γ carbon atoms so that strophanthidin may be designated as a substituted $\Delta\beta$, γ crotonic lactone.[1] Characteristic for strophanthidin and all of its derivatives which still possess the double bond of the parent substance is their behavior in dilute pyridine solution towards Tollens' reagent. For example, pseudostrophanthidin, anhydrostrophanthidin and its ethylal, dianhydrostrophanthidin and its ethylal, and the dilactone $C_{23}H_{26}O_4$, obtained by oxidation of dianhydrostrophanthidin, reduce Tollens' reagent more or less readily. On the other hand the analogous derivatives of dihydrostrophanthidin, in which the olefinic linking has been removed by hydrogenation, no longer react with this reagent or to an extent which is practically negligible in comparison with the behavior of the unsaturated compounds. This is quite striking even in the case of derivatives of dianhydrodihydrostrophanthidin which possess double bonds elsewhere in the molecule than in the lactone ring. Similarly, isostrophanthidin, which results from a shift in the double bond of strophanthidin, only after long standing gradually develops a reaction with this reagent. This test has given such concordant results with all of the substances studied that there appears to be complete justification in attributing the reduction of Tollens' reagent directly to the unsaturated lactone group.

Results of a similar and most striking character have more recently

[1] Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxiv, 383; 1925, lxv, 493.

TABLE I.

Reaction with Tollens' Reagent.

Strophanthidin	+
Dihydrostrophanthidin	−
Dianhydrostrophanthidin	+
Ethylal of oxidodianhydrostrophanthidin	+
" " oxidodianhydrostrophanthidinic acid	+
" " dianhydrodihydrostrophanthidin	−
Dianhydrodilactone, $C_{23}H_{26}O_4$*	+
Dianhydrodiacid, $C_{23}H_{30}O_6$†	+
Tetrahydrodianhydrodilactone, $C_{23}H_{30}O_4$†	+
Hexahydrodianhydrodilactone, $C_{23}H_{32}O_4$‡	−
Pseudostrophanthidin	+
Isostrophanthidin	−
Ouabain	+
Dihydroouabain	−
Gitoxin	+
Digitoxin	+
Bufagin	+

* Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxv, 501.
† Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxv, 503.
‡ Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxv, 504.

TABLE II.

Reactions with Sodium Nitroprusside.

Strophanthidin	+
Dihydrostrophanthidin	−
Dianhydrostrophanthidin	+
Dianhydrostrophanthidinic acid	−
Ethylal of oxidodianhydrostrophanthidin	+
" " oxidodianhydrostrophanthidinic acid	−
" " dianhydrodihydrostrophanthidin	−
Dianhydrodilactone, $C_{23}H_{26}O_4$	+
Dianhydrodiacid, $C_{23}H_{30}O_6$	−
Tetrahydrodianhydrodilactone, $C_{23}H_{30}O_4$	+
Hexahydrodianhydrodilactone, $C_{23}H_{32}O_4$	−
Pseudostrophanthidin	+
Isostrophanthidin	−
Ouabain	+
" saponified	−
Dihydroouabain	−
Gitoxin	+
" saponified	−
Digitoxin	+
" saponified	−
Bufagin	−

been obtained by the use of the nitroprusside test. With this reagent strophanthidin in dilute pyridine solution after addition of a few drops of alkali at once gives a deep red color. A similar positive reaction was given by all derivatives of strophanthidin in which the unsaturated lactone group is still present. But as soon as this group was hydrogenated or lost by saponification to the acid the resulting substances no longer gave a positive reaction.

In the course of studies with ouabain, or gratus strophanthin, which had been initiated in this laboratory a number of years ago and which had been temporarily interrupted by the pressure of other work, the unsaturated nature of this substance was ascertained. Ouabain on hydrogenation was found to absorb 1 mol of hydrogen with the formation of a dihydro derivative.[2] These experiments will be described more fully in a communication which will shortly appear. The early work of Arnaud[3] had shown that the glucoside is converted into an acid, ouabaic acid, by the action of hot alkali, a fact which demonstrates its lactone nature. Ouabain is thus unsaturated and at the same time a lactone. Because of the experience with strophanthidin it was of interest to study the behavior of ouabain and its dihydro derivative towards Tollens' reagent and sodium nitroprusside. A striking difference in the behaviors of the two substances was at once observed which was exactly analogous to the contrast noted in the behaviors of strophanthidin and its hydro derivative. Ouabain reduces Tollens' reagent fairly readily and gives a positive nitroprusside reaction, while its hydrogenation product failed to react with these reagents. Similarly after saponification, ouabain no longer gave a color reaction with sodium nitroprusside. It appears therefore strongly indicated that we are dealing with phenomena analogous to those observed with strophanthidin and its derivatives and that ouabain like this substance contains an unsaturated lactone group. The fact that ouabain absorbs 1 mol of hydrogen on hydrogenation is of interest from another standpoint. It we accept the formula of

[2] In the preliminary note (Jacobs, W. A., and Hoffman, W., *Proc. Soc. Exp. Biol. and Med.*, 1925, xxiii, 214) the statement that ouabain is hydrogenated with the formation of a tetrahydro compound should be corrected as given in this paper.

[3] Arnaud, M., *Compt. rend. Acad.*, 1898, cxxvi, 1280.

this glucoside derived by Arnaud, $C_{30}H_{46}O_{12}$,[4] which Thoms[5] has substantiated and which our own analyses have confirmed, this substance is a rhamnoside of an aglucone, $C_{24}H_{36}O_8$. Since it is unsaturated and contains no carbonyl group, the saturated hydrocarbon of reference is $C_{24}H_{42}$. Since this differs from a paraffin hydrocarbon $C_{24}H_{50}$ by eight hydrogen atoms it can be only tetracyclic. The ouabain aglucone, although a C_{24} derivative, would appear therefore to belong to the tetracyclic group of C_{24} compounds which contains the digitalis aglucones and bufotalin. Strophanthidin, although a tetracyclic compound, is a C_{23} derivative.

The suggestive character of the ouabain tests have caused us to turn to other substances of this pharmacological group, which have been shown to be lactones. As a result of the recent very important contributions of Windaus[6] and his coworkers, the digitalin verum of digitalis seeds which had been the subject of much previous study by Kiliani, and the sparingly soluble anhydrogitalin of Kraft[7] (gitoxin of Windaus and Schwarte) obtained from the leaves have been shown to be in all likelihood glucosides of the same aglucone, gitoxigenin, $C_{24}H_{36}O_5$. This substance was found to be a lactone and also to contain a double bond. We have prepared gitoxin from digitalis leaves and have studied its behavior towards both Tollens' reagent and sodium nitroprusside. In both cases definitely positive reactions were obtained. After gentle saponification with alkali gitoxin no longer gave a positive nitroprusside reaction. Here again there is a strong indication that gitoxigenin like strophanthidin possesses an unsaturated lactone group.

Still another substance which merited similar study is the easily crystallized and soluble glucoside of digitalis leaves, digitoxin, of which the aglucone, digitoxigenin, has been shown by Kiliani to be also a lactone. This worker, however, was unsuccessful in attempts to hydrogenate this substance.[8] From the recent very careful studies

[4] Arnaud, M., *Compt. rend. Acad.*, 1888, cvi, 1013.

[5] Thoms, H., *Ber. pharm. Ges.*, 1904, xiv, 114. ·

[6] Windaus, A., and Bandte, G., *Ber. chem. Ges.*, 1923, lvi, 2001. Windaus, A., Bohne, A., and Schweiger, A., *Ber. chem. Ges.*, 1924, lvii, 1386. Windaus, A., and Schwarte, G., *Ber. chem. Ges.*, 1925, lviii, 1515.

[7] Kraft, F., *Arch. Pharm.*, 1912, ccl, 118.

[8] Kiliani, H., *Ber. chem. Ges.*, 1918, li, 1631.

of Cloetta[9] the formula of digitoxigenin is in all probability $C_{24}H_{36}O_4$. If this formula is accepted and if also the view is adopted that digitoxigenin is a tetracyclic dihydroxylactone, a simple calculation makes it apparent that digitoxigenin must be derived from a hydrocarbon, $C_{24}H_{40}$, which if tetracyclic must contain one double bond. Digitoxigenin would then appear to be a lactone with an unsaturated group.[10] This conclusion was supported by the behavior of the substance towards Tollens' reagent and sodium nitroprusside. Here again positive reactions were obtained and after saponification with alkali a positive reaction with nitroprusside was no longer obtained.[11] It would appear that digitoxigenin also possesses an unsaturated lactone group.

A summary of the results of these observations is briefly given in the accompanying tables. The tests were made as follows: About 10 to 20 mg. of the substance were dissolved in 1 cc. of pure pyridine and diluted with an equal volume of water. The solution was treated with 0.5 cc. of Tollens' reagent and then allowed to stand at room temperature. The reaction occurred gradually as a rule. Definite deposition of silver was apparent during the first 30 minutes at a rate which varied greatly with the individual substance. Those substances which are recorded with negative reactions did not show an appreciable deposit of silver after several hours. In the case of the nitroprusside test a few drops of 10 per cent sodium hydroxide solution were added to the dilute pyridine solution followed by 1 cc. of 0.3 per cent sodium nitroprusside solution. If a positive reaction was given this was shown at once by the appearance of a deep red color. When negative a yellow color only was observed.

It will be of interest to extend these studies to other "genins" belong-

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[9] Cloetta, M., *Arch. exp. Path. u. Pharmakol.*, 1920, lxxxviii, 133.

[10] Since this communication has been sent to press the last paper of Windaus and Freese (*Ber. chem. Ges.*, 1925, lviii, 2503) has appeared in which the presence of one double bond in digitoxigenin was demonstrated directly by hydrogenation experiments.

[11] In the case of ouabain, gitoxin, and digitoxin the apparent association of the double bond and the lactone group was made even more certain by the fact that if the solutions obtained by saponification with alkali, which no longer gave a positive nitroprusside test, were reacidified and heated to permit relactonization, such solutions again gave a positive nitroprusside test.

ing to this general group of cardiac poisons, such as antiarigenin, scillarigenin, bufotalin, and the like. Through the kindness of Dr. J. J. Abel we have already obtained a small sample of bufagin, the poison isolated by Abel and Macht[12] from the tropical toad, *Bufo agua.* This substance was found to reduce Tollens' reagent very promptly, but in its behavior towards sodium nitroprusside it proved to be an exception to the rule. But the significance of this failure to give a positive nitroprusside test cannot be evaluated in view of our very meager knowledge of the chemistry of the substance.

Of very great importance in connection with the results here reported are the interesting observations of Windaus, Bohne, and Schwieger[13] with regard to the influence of hydrogenation on the toxicity of digitalin. Whereas 0.5 mg. of digitalinum verum proved to be a lethal dose for a 35 gm. frog, 6 and 8 mg. of the dihydro derivative proved to be non-toxic. We have made a few crude similar toxicity tests on frogs to compare the effect of hydrogenation on ouabain. Whereas 0.02 mg. of ouabain proved to be a lethal dose, frogs of about 40 gm. weight just tolerated doses of 2 mg. or 100 times as much dihydroouabain. It is not excluded that the amorphous dihydro compound which we have employed may still have contained traces of unchanged ouabain. At any rate these experiments suggest that the unsaturated group of these compounds may be essential for their pharmacodynamic effect. It will be of interest to confirm this by the further study of the effect of hydrogenation on the toxicity of other substances of this group.

Although the observations which have been reported here may not be construed as conclusive evidence, nevertheless they are strongly suggestive of the probability that the aglucones of ouabain, of the digitalis glucosides, and perhaps of other substances of this pharmacological group, possess, like strophanthidin an unsaturated lactone group and that this group may be essential, perhaps in conjunction with other structural features, for the pharmacodynamic action of these substances. We are at present attempting to ascertain by more direct chemical methods, as has already been accomplished in the

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[12] Abel, J. J., and Macht, D. I., *J. Pharmacol. and Exp. Therap.*, 1911–12, iii, 334.

[13] Windaus, A., Bohne, A., and Schwieger, A., *Ber. chem. Ges.*, 1924, lvii, 1388.

case of strophanthidin, whether these substances are indeed inner esters of enolized ketones.   An attempt will also be made to confirm if possible the suggested responsibility of this unsaturated lactone group for the cardiac action of this group of poisons by the synthesis and pharmacological study of simpler substances containing unsaturated lactone groups.

ACTION DU PRINCIPE FILTRANT D'UN SARCOME DU GOUDRON SUR DES CULTURES DE RATE.

Par ALEXIS CARREL.

(*Des laboratoires, The Rockefeller Institute for Medical Research, New York.*)

Dans une note précédente (1), j'ai exposé que l'extrait filtré d'un sarcome obtenu chez une poule par la transplantation d'un fragment de tumeur du goudron produisit, dans des cultures de rate, une modification morphologique analogue à celle qui est déterminée par le principe de Rous. Ce même extrait, injecté à des poules, provoqua la formation de tumeurs qui tuèrent rapidement les animaux. Les cultures traitées par le principe filtrant du sarcome du goudron et par celui de Rous présentaient un aspect analogue, mais non semblable. Il n'y avait pas identité entre les effets des deux extraits. Je me suis proposé, dans cette note, d'examiner ce point d'une manière plus détaillée.

Le sarcome fusocellulaire dont l'extrait filtré servit à ces expériences, appartenait au "Mc Faul strain," qui produit des tumeurs d'une grande malignité, tuant parfois les poules en 4 ou 5 jours. Cette tumeur est la plus maligne de toutes celles que nous connaissons. La rapidité de sa croissance, son caractère envahissant, les hémorragies que provoquent ses métastases dans les poumons, sa grande vascularisation, la distinguent du sarcome de Rous qui tue les animaux en deux semaines environ. L'extrait filtré de ce sarcome du goudron détermine la mort des poules plus rapidement que ne le fait le principe de Rous. Nous étudiâmes l'action de cet extrait sur des fragments de rate de fœtus de poulet cultivés dans des flacons D-5 par la technique antérieurement décrite. Comme l'aspect des cultures varia peu, je me contenterai de donner ici la description d'une expérience typique.

Le 15 avril, 1925, on ajouta 0.5 c.c. d'extrait filtré de sarcome Mc Faul au milieu habituel d'une culture de trois fragments de

(1) Carrel. *C. R. de la Soc. de biol.*, 1925, t. xcii, p. 1491.

rate dans un flacon. Dans la culture témoin, on injecta seulement 0.5 c.c. de solution de Tyrode. Cette dernière culture demeura entièrement normale. Des macrophages et des fibroblastes émigrèrent pendant longtemps dans le coagulum. Aucune digestion ne fut observée. Au contraire, la culture contenant le principe filtrant, manifesta dès le 16 avril des changements appréciables. Le premier symptôme de la maladie déterminée par l'agent filtrant fut l'apparition de quelques cadavres de macrophages, isolés ou agglomérés, au milieu des éléments cellulaires envahissant le milieu de culture. Le 24 avril, on constata pour la première fois une petite zone de digestion dans le voisinage d'un des fragments de rate. Cette zone s'étendit les jours suivants. Elle était comme découpée à l'emporte pièce. Son fond atteignait la paroi du flacon. Sa circonférence était régulière et ne présentait ni liséré blanchâtre, ni points opaques. Cependant, à l'aide du microscope, on apercevait quelques petites masses de tissu amorphe. Le 25 avril, la zone de digestion continua à s'agrandir, et le 28 avril, on dut renforcer le coagulum par l'addition de 1 c.c. de plasma dilué. On plaça également dans la région digérée un fragment de rate fraîche. Dès le 30 avril, le coagulum se digéra de nouveau, et il fallut y ajouter du plasma. Cette addition de plasma n'entrava pas le processus de digestion, qui continua avec activité tout en se limitant à deux régions. La zone détruite présentait des bords taillés à pic, réguliers, sans anfractuosités, ni dentelures et on n'observait autour d'elle aucun centre secondaire de raréfaction du milieu. Sa digestion était tellement étendue, le 7 mai, qu'on extirpa un petit fragment du coagulum près de la région digérée, et qu'on le plaça avec deux fragments de rate fraîche, dans un autre flacon. Les cellules contenues dans le lambeau de coagulum et les fragments de rate prolifèrent rapidement. Mais le 11 mai, au milieu des éléments cellulaires émigrant dans le milieu apparut une zone de digestion, qui s'étendit les jours suivants, et ne fut pas arrêtée par l'addition répétée de plasma. · La surface digérée présentait des caractères identiques à ceux que nous avions déjà observés.

Le 19 mai, le coagulum fut retiré du flacon, et inoculé à une poule. Le 31 mai, l'animal présentait au point inoculé, une large tumeur dont il mourut le 18 juin.

Les caractères que je viens de décrire, ont été observés également

dans les autres cultures de rate ou de leucocytes traités par l'extrait filtré de sarcome du goudron. Nous devons les comparer à ceux des cultures traités par le principe filtrant de Rous. Les deux principes filtrants ont le caractère commun fondamental de déterminer la transformation de cellules normales en cellules sarcomateuses. Dans les deux cas, les cultures contiennent des cadavres cellulaires, des zones de digestion et des petits amas de tissu amorphe. Néanmoins, elles ne présentent pas le même aspect. On sait que les cultures de rate ou de leucocytes infectées par l'extrait filtré du sarcome de Rous prennent un aspect très caractéristique. De petits anneaux à contours irréguliers et blanchâtres apparaissent en plusieurs régions de la culture. Ce sont des centres de raréfaction du milieu, autour desquels se trouvent de petits amas de tissu nécrosé. La digestion devient plus profonde en même temps que d'autres centres de nécrose se développent en des points variés du coagulum. La surface du milieu ressemble alors à une carte de géographie, à une mer parsemée d'îles nombreuses à contours déchiquetés, ou bien à une étoffe rongée par les mites. Il n'en est pas de même pour les cultures infectées avec le principe filtrant du sarcome du goudron. La digestion ne se produit qu'en deux ou trois régions du coagulum. Elle creuse de large trous, à bords réguliers et abrupts, qui ne sont pas garnis de masses nécrosées. Leur aspect se rapproche beaucoup des cultures de rate normale traitées par une faible quantité de goudron. Ces différences sont, sans doute, d'importance secondaire, mais elles traduisent peut-être certaines particularités du mode d'action des principes filtrants et de leur constitution.

LE PRINCIPE FILTRANT DES SARCOMES DE LA POULE PRODUITS PAR L'ARSENIC.

Par ALEXIS CARREL.

(*Des laboratoires, The Rockefeller Institute for Medical Research, New York.*)

Dans des notes précédentes (1*), j'ai montré qu'un sarcome fuso-cellulaire du goudron d'une très grande malignité pouvait être propagé facilement par son extrait filtré, et que cet extrait filtré possédait, ainsi que le principe de Rous, la propriété de transformer *in vitro* des macrophages en cellules sarcomateuses et de se reproduire abondamment dans les cultures. Ces expériences conduisirent naturellement à l'hypothèse que le sarcome de Rous n'est nullement infectieux et que l'agent filtrant doit être considéré, non pas comme un virus, ainsi que le croit Gye, mais comme une substance possédant certaines propriétés des virus et fabriquée par les tissus sous l'influence de corps chimiques non spécifiques. Afin de vérifier cette hypothèse, nous avons essayé de produire des sarcomes chez la poule à l'aide de substances variées et de déterminer si les extraits filtrés de ces néoplasmes pouvaient reproduire la tumeur primitive *in vivo* et *in vitro.*

Nous savons depuis longtemps que l'arsenic détermine parfois chez l'homme des tumeurs malignes. Dans un travail de Hutchinson (2*), on peut trouver une excellente description de ces faits. Mais la longueur de la période nécessaire à l'établissement des lésions ne permet pas d'affirmer que l'arsenic soit la cause unique de la tumeur. Je me suis donc proposé d'obtenir une transformation rapide des tissus sains en tissus sarcomateux dans des conditions telles que la possibilité d'une infection surajoutée fut entièrement éliminée. En mai et juin, 1925, nous avons inoculé 16 poules avec un mélange d'acide arsénieux et de pulpe embryonnaire. Chaque animal reçut une seule injection dans les muscles de la paroi thoracique. La quantité d'anhydride arsénieux en solution dans le mélange variait de 1 pour

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(1*) A. Carrel. *C. R. de la Soc. de biol.,* 1925, t. xcii, p. 1491; *C. R. de la Soc. de biol.,* 1925, t. xciii, p. 491.

(2*) J. Hutchinson. *Trans. path. Soc. London,* 1888, t. xxxix, p. 352.

5.000 à 1 pour 250.000. Aucune tumeur ne se développa chez 11 animaux qui reçurent les solutions les plus concentrées, de 1 pour 5.000 à 1 pour 50.000. A 5 poules, on injecta des solutions contenant respectivement 1 pour 125.000, 1 pour 150.000, 1 pour 200.000 et 1 pour 250.000 d'anhydride arsénieux. Dans 4 cas, des tumeurs se développèrent rapidement, devinrent très volumineuses et amenèrent la mort des animaux en 20 jours, 35 jours, 32 jours et 17 jours. La tumeur était un sarcome fusocellulaire. Dans tous les cas, on observa des métastases dans les poumons, dans deux cas dans le foie, et dans un cas dans la rate. Il est important de remarquer qu'une des poules fut tuée 17 jours après l'inoculation d'arsenic par une grosse tumeur et des métastases viscérales. La transformation maligne des tissus se fit très peu de temps après l'inoculation, ainsi que le montre l'observation suivante. Douze jours après l'injection d'arsenic, nous extirpâmes un petit fragment de la tumeur. Ce fragment fut cultivé dans un flacon. La culture présenta bientôt l'aspect d'une culture de sarcome, et fut inoculée quelques jours après à une poule qui mourut rapidement d'une tumeur.

Deux des tumeurs primitives furent transmises par transplantation à des poules. La première de ces tumeurs fut inoculée à 8 poules au cours de trois passages. Tous les animaux moururent. La durée moyenne de la survie fut de 17 jours. La tumeur la plus maligne produisit la mort en 9 jours. On cessa les transplantations après le troisième passage. Au cours de 14 passages, la seconde tumeur fut transplantée à 48 poules, dont 44 sont mortes, tandis que des représentants du 13e et 14e passages sont encore vivants. Quelques animaux moururent en 10 à 12 jours. Mais la plupart survécurent 18 à 20 jours. Ces deux tumeurs sont donc malignes, mais d'une malignité inférieure à celle de notre sarcome du goudron. Plusieurs de ces tumeurs furent cultivées *in vitro*. Les fragments s'entourèrent de cellules amiboïdes et de fibroblastes, dont la vie fut courte. Des zones de digestion apparurent dans le milieu, et les cultures prirent un aspect analogue à celui des cultures de sarcome de Rous.

Un extrait de la première tumeur fut filtré sur Berkefeld et injecté à 4 poules. Ces animaux moururent respectivement au bout de 27, 20, 25 et 19 jours, avec des grosses tumeurs et des métastases

dans les poumons, le foie et la rate. On fit également un extrait
filtré du premier passage de la seconde tumeur et on l'inocula à 3
poules, qui furent atteintes de sarcomes et moururent au bout de
20, 24 et 30 jours. Un extrait filtré du quatrième passage de cette
même tumeur fut inoculé à deux poules qui moururent en 27 et 31
jours. Le sarcome ainsi produit par l'extrait filtré fut propagé par
transplantation à 13 poules qui moururent toutes de 10 à 30 jours
après l'injection. Il est donc évident que les tumeurs arsenicales
contiennent un principe filtrant analogue à celui de la tumeur de
Rous, et capable de produire *in vivo* l'apparition d'un sarcome fuso-
cellulaire.

Les extraits furent inoculés à des cultures de leucocytes. Dans
une expérience fait par Ebeling, une zone de digestion apparut 5
jours après l'inoculation à la culture de 0,5 gr. d'extrait filtré. Huit
jours plus tard, la culture présentait l'aspect ordinaire d'une culture
de sarcome. Inoculée à une poule, elle produisit une tumeur qui
la tua. Dans un autre cas, la transformation caractéristique de la
culture s'observa 6 jours après l'inoculation. Ces faits achèvent
de montrer que l'extrait filtré des sarcomes arsenicaux se comportent
de la même façon que le principe de Rous.

En somme, l'acide arsénieux a produit en quelques jours chez la
poule la transformation des tissus normaux en sarcomes fusocellu-
laires, qui contiennent un principe filtrant analogue à l'agent de
Rous. Il semble donc, que, dans ce cas comme dans celui des sarcomes
très malins du goudron, nous nous trouvions en présence d'un phé-
nomène. important: la formation par les tissus sous l'influence d'une
substance chimique non spécifique d'un principe qui ressemble à un
virus, agit de façon spécifique sur des cellules d'un certain type, et se
reproduit indéfiniment en présence de ces cellules.

UN SARCOME FUSOCELLULAIRE PRODUIT PAR L'INDOL ET TRANSMISSIBLE PAR UN AGENT FILTRANT.

Par ALEXIS CARREL.

(Des laboratoires, The Rockefeller Institute for Medical Research, New York.)

Une poule reçut dans le muscle pectoral droit, 4 c.c. de pulpe embryonnaire contenant approximativement 1 p. 1.000 d'indol, et dans le muscle pectoral gauche la même quantité de pulpe contenant de 1 pour 1.250 à 1 p. 5.000 d'indol. On répéta cette dernière injection sur deux autres poules. Une quatrième poule reçut dans chaque pectoral 4 c.c. de pulpe et d'indol aux concentrations de 1 p. 2.000 et de 1 p. 20.000. Chez la seconde et la troisième poule apparurent rapidement des tumeurs, qui envahirent en deux mois toute la paroi latérale du thorax. Les deux animaux moururent pendant l'été à un moment où l'autopsie ne put être pratiquée. Quant à la quatrième poule, elle présenta deux tumeurs, l'une, moyenne, correspondant à l'injection d'indol à 1 p. 2.000, et l'autre, volumineuse, correspondant à la solution la plus concentrée. La première tumeur rétrocéda bientôt tandis que la seconde atteignait en 3 mois un volume considérable. Elle se composait surtout de kystes, d'os, et de cartilage. L'animal demeura en bonne santé.

L'histoire de la première poule fut beaucoup plus intéressante. Du côté droit, aucune tumeur ne se développa. Du côté gauche, on aperçut bientôt une tumeur qui atteignit rapidement une longueur de 11 cm., une largeur de 9 cm., et une épaisseur de 5 cm. environ. Au bout de 26 jours, la poule était mourante. La paroi thoracique était infiltrée par un tissu friable, ressemblant au sarcome de Rous. Nous trouvâmes de métastases dans les poumons, la rate, le foie et le péritoine. Les coupes montrèrent qu'il s'agissait d'un sarcome fusocellulaire. Des fragments de ce sarcome furent à plusieurs reprises cultivés dans des flacons D-5. Ils s'entourèrent rapidement de cellules amiboïdes à vie courte. Ensuite des fibroblastes émigrèrent dans le coagulum. Des zones de digestion se produisirent et la culture prit l'aspect caractéristique des sarcomes de Rous, du goudron et de l'arsenic.

La tumeur se propagea facilement par transplantation. De juin à octobre, le sarcome subit 12 passages chez 54 poules qui, sauf les deux dernières, sont mortes porteuses de tumeurs volumineuses. On pratiqua l'autopsie de 30 poules seulement. Tous les animaux, excepté deux, présentaient des métastases dans les poumons, le foie ou la rate, et parfois dans les trois organes simultanément. Les animaux ne servécurent pas longtemps à la transplantation. La tumeur primitive, qui tua l'animal en 26 jours, fut inoculée à 3 poules qui moururent respectivement en 9 jours, 7 jours et 3 jours. La survie moyenne ne fut donc que de 6,3 jours. La survie moyenne des poules qui reçurent la tumeur à son 2e passage fut de 12,2 jours. Au 3e passage, la survie atteignit 14 jours et au 8e passage, 15 jours. Quoique la tumeur soit encore très maligne, elle n'a pas récupéré l'activité qu'elle présentait à son premier passage.

Nous recherchâmes ensuite si la tumeur pouvait être transmise par un agent filtrant. Un extrait aqueux de la tumeur primitive fut filtré dans un filtre Berkefeld et inoculé à 3 poules qui furent atteintes de grosses tumeurs de la paroi thoracique et moururent au bout de 24 et 30 jours avec des métastases dans le poumon, la rate, le foie et le cœur. Cette expérience fut répétée avec une des tumeurs du premier passage. Les 5 poules inoculées survécurent respectivement 13 jours, 26 jours, 22 jours et 19 jours à l'inoculation, et l'autopsie montra la présence de grosses tumeurs locales et de métastases du poumon, du foie et du cœur. L'extrait filtré d'un des sarcomes du 3e passage détermina, chez la seule poule à laquelle il fut injecté, une grande tumeur et des métastases dans le foie et la rate. La poule mourut au bout de 25 jours. Tandis que l'extrait filtré du sarcome a toujours déterminé l'apparition d'une tumeur maligne chez les poules, il n'a pas encore produit *in vitro* la transformation de monocytes normaux en cellules sarcomateuses, et il ne s'est pas multiplié dans le milieu de culture. L'insuccés des onze expériences faites jusqu'à présent ne prouve pas que l'agent filtrant soit incapable de déterminer *in vitro* la transformation sarcomateuse des leucocytes. Les expériences sont encore en trop petit nombre et doivent être répétées avant que nous puissions savoir si le sarcome de l'indol diffère sur ce point des autres sarcomes d'origine chimique. Il faut remarquer que, dans un cas, Ebeling inocula de l'extrait centri-

fugé et non filtré d'un sarcome de l'indol à des cultures de leucocytes, et que la transformation maligne se produisit.

Il est intéressant de constater que l'indol, substance existant dans l'organisme normal et produite également par les microbes, est capable de déterminer, dans certaines conditions, la transformation maligne de tissus normaux.   On conçoit donc que, grâce à cette substance ou à des substances analogues, un sarcome puisse aussi bien apparaître de façon spontanée dans l'organisme qu'être engendré par des parasites ou des microorganismes variés. Ce fait établit une relation précise entre les théories parasitaire et chimique de l'origine des tumeurs. La transmissibilité de ce sarcome par un agent filtrant vient à l'appui de l'hypothèse que j'ai exposée dans une note précédente (1).   Il est vraisemblable que la production du virus de Rous et du sarcome fusocellulaire par les tissus de la poule constitue la seule réponse que puissent faire ces tissus à des substances aussi différentes que l'arsenic et l'indol, de même que l'inflammation banale est l'unique réaction de l'organisme à des agents très hétérogènes tels que le silicate de soude, le staphylocoque et la térébenthine.

(1) A. Carrel. Le principe filtrant des sarcomes de la poule produits par l'arsenic. *C. R. de la Soc. de biol.*, 1925, t. xciii, p. 1083.

UN SARCOME DU GOUDRON DE FAIBLE MALIGNITE ET TRANSMISSIBLE PAR SON EXTRAIT FILTRE.

Par ALEXIS CARREL.

(*Des laboratoires, The Rockefeller Institute for Medical Research, New York.*)

Dans une note précédente (1), j'ai mentionné une expérience dans laquelle, une poule ayant reçu d'un côté du corps une injection de pulpe d'embryon et de l'autre côté un peu de goudron, une tumeur se développa dans la pulpe embryonnaire. Des cultures de rates inoculées avec de l'extrait filtré de cette tumeur présentèrent certains caractères propres aux cultures de sarcome. Mais leur inoculation à des poules ne détermina pas l'apparition de tumeurs. Comme la fin de cette expérience, qui n'est pas décrite dans la note précédente, est intéressante, je crois utile de donner une description plus complète des phénomènes observés.

Le 19 décembre 1924, on injecta dans les muscles pectoraux du côté gauche d'une poule, 1 c.c. d'éther contenant 25 p. 100 de goudron. Une seconde injection de goudron fut faite le 31 janvier 1925. Les muscles du côté droit reçurent 3 c.c. de pulpe embryonnaire mêlée à une très petite quantité de kieselguhr.

Une petite tumeur apparut du côté droit au point de l'injection de pulpe embryonnaire. Le 11 février, elle était devenue un embryome volumineux, tandis qu'aucune tumeur ne s'était développée dans la région qui avait reçu les deux injections de goudron. Cette tumeur fut extirpée en partie et inoculée à 5 poules, et récidiva. Le 9 mars, quand la poule fut tuée; on ne trouva aucune métastase viscérale.

Chez une seule des 5 poules inoculées, une tumeur se développa. On l'extirpa le 19 février. Une partie fut inoculée à 4 poules, mais aucune tumeur ne se produisit. Une autre partie fut réduite en pulpe, mêlée à une quantité égale de solution de Tyrode, et centrifugée. Le liquide fut additionné de 9 volumes de solution de Tyrode et filtré dans un filtre Berkefeld. L'extrait filtré fut mêlé à de la pulpe d' embryon et injecté à 4 poules.

(1) A. Carrel. *C. R. de la Soc. de biol.*, 1925, t. xciii, p. 1491.

Chez 3 animaux, les résultats de l'inoculation furent négatifs. L'histoire de la 4ᵉ poule peut être résumée de la façon suivante. Le 19 février, on injecta dans les muscles pectoraux gauches un mélange d'extrait filtré et de pulpe embryonnaire, en parties égales. Le 25 février, on sentit une petite tumeur dans le muscle. Le 28 mars, la tumeur avait atteint le volume d'un petit œuf. Le 15 avril, elle avait un peu augmenté, et le 19 mai, la poule, qui était en excellente santé, mourut subitement.

Dans la paroi thoracique gauche, on trouva une tumeur ayant infiltré le tissu musculaire et présentant l'aspect d'un chondro-sarcome. Dans certaines parties, la tumeur ressemblait au sarcome de Rous. L'examen histologique de ces régions montra qu'il s'agissait d'un sarcome fusocellulaire, il n'y avait aucune métastase viscérale. On trouva une petite tumeur adhérente aux vertèbres lombaires, et la base du cou était occupée par une grosse tumeur qui bloquait la partie supérieure du thorax et qui avait probablement tué l'animal par compression de la trachée. Cette tumeur avait le volume d'un œuf de pigeon et adhérait à la partie antérieure des vertèbres cervicales. Sa constitution était celle d'un sarcome fusocellulaire.

En résumé, deux inoculations d'une solution éthérée de goudron d'un côté du thorax déterminèrent la production d'une tumeur dans la pulpe embryonnaire injectée de l'autre côté. Cette tumeur inoculée à 4 poules ne détermina pas le développement d'un sarcome. Mais son extrait filtré injecté avec de la pulpe embryonnaire à 4 poules produisit dans un cas un sarcome fuso-cellulaire qui tua l'animal par une métastase cervicale. Cette tumeur paraît donc être une forme intermédiaire entre les tumeurs de Murphy et Landsteiner (2) qui ne furent pas transmises par leur extrait filtré et le sarcome très malin du goudron, que nous avons déjà mentionné, et dont l'extrait filtré tua toutes les poules inoculées (1).

(2) J. B. Murphy et K. Landsteiner. *Journ. of exp. Med.*, 1925, t. xli, p. 807.

CARBON DIOXIDE PRODUCTION AND DURATION OF LIFE OF DROSOPHILA CULTURES.

BY JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

One of the most concrete and plausible mechanisms which have been proposed for the regulation of the duration of life is what may be termed the *energy limit*, elaborated by Rubner.[1] Rubner stated that the total energy transformed per kilo of body weight during the total life of the animal was approximately constant for a large number of animals. He suggested, therefore, that the duration of life of the individual was determined by the time required to transform this quantity of energy. Slonaker[2] found that 4 albino rats which were allowed to exercise freely died sooner than others which were allowed only limited exercise, and this has been considered by Pearl[3] as additional evidence in favor of Rubner's view. It was found by Loeb and the writer[4] that the duration of life of aseptic *Drosophila* cultures was a function of temperature, the insects living longer at a low temperature. Since they are also more sluggish at a lower temperature Pearl has suggested that this also is evidence in favor of the energy limit. There is further confirmation of the idea in the fact, as pointed out by Crozier,[5] that the temperature coefficients of the duration of life and of the rate of oxidation may be similar. No direct measurements comparing the total energy production with the duration of life have been made, and the present experiments with *Drosophila* were designed to test this point. It has been found that there is considerable variation in the total amount of CO_2 produced by cultures of *Drosophila*

[1] Rubner, M., Das Problem der Lebensdauer, München and Berlin, 1908.

[2] Slonaker, J. R., *J. Animal Behavior*, 1912, ii, 20.

[3] Pearl, R., The biology of death, Monographs on experimental biology, Philadelphia and London, 1922, 213.

[4] Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1917, xxxii, 103.

[5] Crozier, W. J., *J. Gen. Physiol.*, 1924–25, vii, 189.

during their entire life under different conditions, and that, therefore, the duration of life of the insect is not determined by the time required to transform a definite amount of energy.

Methods.

The flies used in these experiments were taken from the 195th to the 205th generation of aseptic cultures used by Loeb and the writer.[4] These flies have been inbred from the original pair of aseptic flies; the cultures have been kept in the dark at room temperature and raised on sterilized yeast. It will be noted that the duration of life differs markedly from that given previously. A progressive decrease
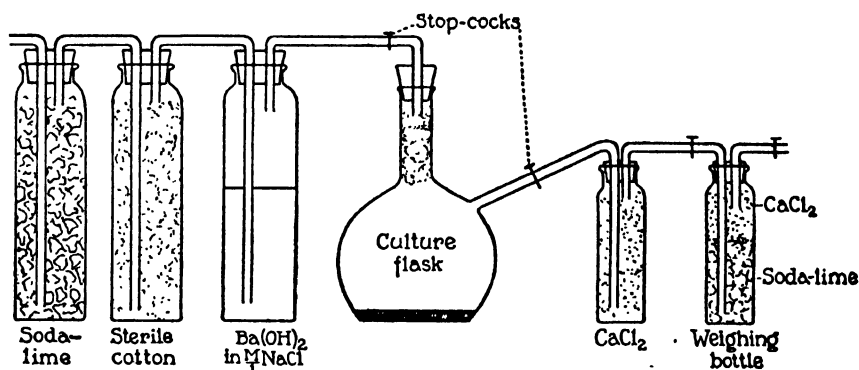
FIG. 1. Apparatus for CO₂ determination.

in the duration of life of these cultures, especially at low temperatures, had been noted for several years.

Determination of CO₂.

The arrangement of the apparatus for the determination of CO_2 is evident from Fig. 1. The cultures were connected to a soda-lime weighing tube as shown in the figure. A slow current of air was blown through the flask every 2 or 3 days for 3 hours and the CO_2 weighed. The number of larvæ, pupæ, or imagos was also counted, and the CO_2 produced per 100 individuals was calculated. A control flask containing no insects, run simultaneously, showed no increase in CO_2. The culture flasks themselves were also continued after all the insects were dead. After the death of the insects no CO_2 was found, showing

that the apparatus was air-tight and that the CO_2 in the air stream had been completely removed by the soda-lime. The larvæ were grown on sterilized yeast as already described and the imagos were kept on glucose agar. Any flasks showing contamination with micro-organisms were discarded.

The 15°, 26°, and 30°C. cultures were kept in the dark in water-jacketed incubators, and the 22–26°C. light culture was kept at the room temperature, in diffuse daylight, and in addition illuminated with a 40 watt bulb attached to a circuit breaker so that the light was turned on and off at irregular intervals. This culture was also disturbed more or less by persons working in the laboratory and the flies were much more active than those in the dark. Each culture contained from 300 to 400 flies.
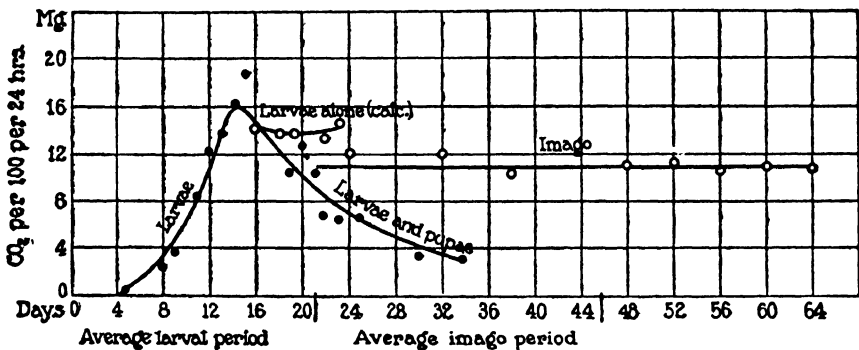
FIG. 2. Production of CO_2 per 100 individuals per day, in dark, at 16°C.

The result of a complete experiment is shown in Fig. 2, in which the CO_2 per 24 hours per 100 individuals has been plotted against the time in days. The curve confirms the results of Fink,[6] in that it shows a rapid increase during the larval period followed by a decrease in the pupal period. Since the curve is per individual and not per unit of weight, the rise is sharper than in the CO_2 per unit weight curve as it includes the increase in size of the larvæ. It will be noted that the curve of CO_2 production of the imagos is practically constant throughout life. This result was obtained in every experiment, the only decrease noted being at the end of the experiment when the number of

[6] Fink, D. E., *J. Gen. Physiol.*, 1924–25, vii, 527.

TABLE I.

Duration of Life and CO₂ Production of Drosophila Cultures under Various Conditions.

Larvae.

Experiment.	Temperature, etc.	Average duration of larval period.	Average CO₂ per 24 hrs. per 100.	Total CO₂ per 100 larvae.
	°C.	days	mg.	mg.
1/8	15 Dark.	21	8.0	170
3/17	15 "	20	7.5	150
2/19	26 "	6.3	13.0	83
2/19a	26 "	6.1	13.4	82
1/9	26 "	6.2	12.0	73
1/9	22–26 Light.	6.5	10.8	70
2/11	22–26 "	7.2	12.0	87
2/19	22–26 "	6.6	17.0	112 94
2/19	22–26 "	7.3	15.0	108
3/16	30 Dark.	4.6	14.0	66
2/19	30 "	6.1	15.4	94

Imagos.

Experiment.	Temperature, etc.	Average duration of life.	Average CO₂ per 100 imagos per 24 hrs.	Total CO₂ per 100 imagos.	Total CO₂ for entire duration of life.
	°C.	days	mg.	mg.	mg.
2/10	16 Dark.	24	11.4	275	445
4/15	16 "	26	11.1	290	440
4/29	26 "	13	14.5	189	
5/17	26 "	9.3	12.0	110 192	272
4/15	26 "	9.0	29.0	260	
5/17	26 "	9.2	23.0	210	
3/2a	22–26 Light.	15.0	22.0	330	
3/2b	22–26 "	16.0	21.0	331 317	411
3/31	22–26 "	15.2	18.0	275	
3/31b	22–26 "	15.0	22.0	330	
3/30	30 Dark.	7.6	19.6	149	
2/26	30 "	8.1	17.5	141 166	246
3/9	30 "	11.9	17.6	210	

flies was very small and the error proportionately large. There is, therefore, no evidence of any "running down" as might have been expected were the duration of life determined by the transformation of a limiting amount of energy.

A summary of all the experiments is given in Table I. The experiments show that the total amount of CO_2 produced by the insects during the larval, the imaginal, or during the entire duration of life varies considerably with the conditions.[7] More CO_2 is produced at 15° than at 26° when both cultures are in the dark; $i.e.$, the temperature coefficient of CO_2 production is smaller than that for the duration of life or the duration of the larval period. The cultures which were exposed to the light, however, produced much more CO_2 than those in the dark.[8] This effect of light on CO_2 production is well known, and was shown by Loeb[9] to be due to an increase in muscular activity, since insect pupæ are not affected. At 30°C., in the dark, there is a still further decrease in the total CO_2, owing to the fact that the CO_2 per day remains nearly constant while the rate of growth and duration of life is shortened. 30°C. is above the normal temperature range of the insect since successive generations cannot be reared at this temperature, and it is possible that the results at this temperature are not significant on this account, since it is evident that death due to injury cannot be determined by energy limitations.

The results are corroborated by the fact that quite high light intensities have no effect on the duration of life of these insects,[10] whereas numerous investigators have shown that illumination markedly increases the CO_2 production.

SUMMARY.

The total CO_2 produced by aseptic *Drosophila* cultures during the entire duration of life has been determined at 15°, 26°, and 30°C. in the dark and at 22–26°C. in the light.

[7] Owing to experimental difficulties the CO_2 production of the pupæ has been omitted. It is very small compared to that of either larvæ or imagos.

[8] The longer duration of life of the light cultures is due to the slightly lower temperature.

[9] Loeb, J., *Arch. ges. Physiol.*, 1888, xlii, 393.

[10] Northrop, J. H., *J. Gen. Physiol.*, 1925–26, ix, 81.

The total amount of CO$_2$ produced is not constant but is greater at 15° than at 26° or 30°, and is much greater in the light than in the dark.

The total duration of life, therefore, is not determined by the time required to produce a limiting amount of CO$_2$.

THE COMBINATION OF SALTS AND PROTEINS.

II. A METHOD FOR THE DETERMINATION OF THE CONCENTRATION OF COMBINED IONS FROM MEMBRANE POTENTIAL MEASUREMENTS.

BY JOHN H. NORTHROP AND M. KUNITZ.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The exact determination of the combination of proteins with the ions of electrolytes has been limited practically to those cases in which concentration cells can be used. Bugarszky and Liebermann[1] found by this method that the hydrogen ion is very largely combined and chloride to a much less extent. These measurements have been repeated and confirmed by a number of workers, and extended by Pauli and Matula[2] to Ag^+, which was found to be also quite largely combined. Pauli and Samec[3] also found that the solubility of slightly soluble salts was increased by proteins, and this furnishes an additional method for determining the amount of combined ions. Both methods, however, are limited to a very small number of ions and only in rare cases, such as HCl or $ZnCl_2$, is it possible to determine the combination of both ions. It was pointed out in the preceding paper[4] that the Donnan equilibrium furnishes a general method which can be used for any ion, provided the distribution of one ion and the analytical composition of the solutions be known. It was shown that using gelatin particles and determining the theoretical ion ratio from Cl^- electrode potentials, the concentration of Zn ions combined with the gelatin could be calculated, and agreed quite well with the values determined directly by concentration cell measurements. This method is also limited since it can only be used with gelatin and under certain conditions, since in alkaline solutions the chloride electrodes

[1] Bugarszky, S., and Liebermann, L., *Arch. ges. Physiol.*, 1898, lxxii, 51.
[2] Pauli, W., and Matula, J., *Biochem. Z.*, 1917, lxxx, 187.
[3] Pauli, W., and Samec, M., *Biochem. Z.*, 1909, xvii, 235.
[4] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1924–25, vii, 25.

cannot be used.   It is evident from Donnan's theory that the desired potential is also given by the membrane potential, so that if this be accurately measured the ratios of the activities of all the ions inside and outside of the membrane can be calculated.   Loeb[5] showed, by comparing the membrane potential with the hydrogen and chloride electrode potential, that the theory was completely borne out by experiment.   If, therefore, a protein solution in a membrane be allowed to come to equilibrium with an electrolyte solution, and the membrane potential and ion concentrations determined, the effect of the protein on the activity of the various ions can be calculated.   This furnishes a general method which can be used for any ion.

Theoretically the procedure is very simple, but experimentally there are a number of difficulties and several important sources of error.   It is essential that the system be at equilibrium and also that no diffusion potentials enter at the various liquid junctions.   It is impractical to measure the potential while the system is in osmotic equilibrium and it is necessary to show that the potential is not affected by removing the pressure.   Agreement between electrode and membrane potential may be used as a test for all these errors, since the P. D. will agree only when these errors are eliminated.   It was found possible, after considerable difficulty, to regulate conditions so that the membrane potential agreed closely with the electrode potentials, and the combined ions calculated from this membrane potential agreed with the results obtained from concentration cells.

Experimental Procedure.

The experiments were carried out with 10 per cent gelatin solution, at 37°C., using collodion membranes.

Preparation of the Membranes.—In order to avoid stretching of the membrane and the passage of small amounts of protein through it, it was found necessary to standardize conditions during the preparation.   The following procedure was found to produce membranes which stretch very slightly if at all during the experiment and which were practically impermeable to gelatin at 37°C.   They were used only once, since after the experiment they become very impermeable even to electrolytes.

7.5 cc. of Merck's U.S.P. collodion (4 per cent dry weight) were placed in a 175

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[5] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

× 20 mm. test-tube and the tube rotated mechanically in a nearly horizontal position at about 10 rotations per minute for 5 minutes, care being taken to prevent the collodion from running out over the lip of the tube. Air, under 1 cm. Hg pressure, was then blown from two 1.5 mm. tubes inserted so as to reach, one about half way, and the second nearly to the bottom of the test-tube. This was continued while the tube rotated for about 6 minutes longer depending on atmospheric conditions. It was then put in water and the membrane removed. The
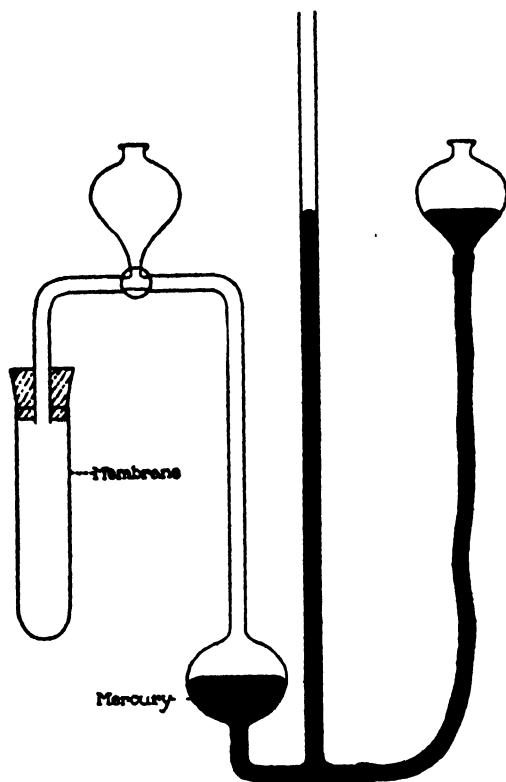
FIG. 1. Apparatus for testing membranes.

membrane was then attached to a rubber stopper by means of rubber bands, and filled with water. 20 cm. Hg pressure was then applied, and the membrane carefully examined for leaks. This pressure causes considerable stretching, so that no further stretching occurs during the experiment. At the same time the rate of flow of the water through the membrane was measured. This serves as a measure of the permeability, and the time of drying was so regulated as to keep this constant. The apparatus shown in Fig. 1 was found very convenient for this purpose.

Preparation of the Gelatin.—Powdered gelatin was washed in dilute alkali, then in dilute acetic acid, and finally at pH 4.7 with ice water, until the gelatin reached a specific conductivity of less than 5×10^{-5} reciprocal ohms. The resulting swollen particles were then melted. Such preparations usually contained about 17 gm. dry weight of gelatin per 100 cc.

Assembling the Apparatus.—Since the final calculation requires an accurate figure for the concentration of electrolyte, both in the gelatin and in the surrounding liquid, it is necessary to know the water content of the solution with considerable accuracy. This can best be done by weight. The concentrations, therefore, in this paper are all expressed as molal; *i.e.*, the number of mols of solute per 1,000
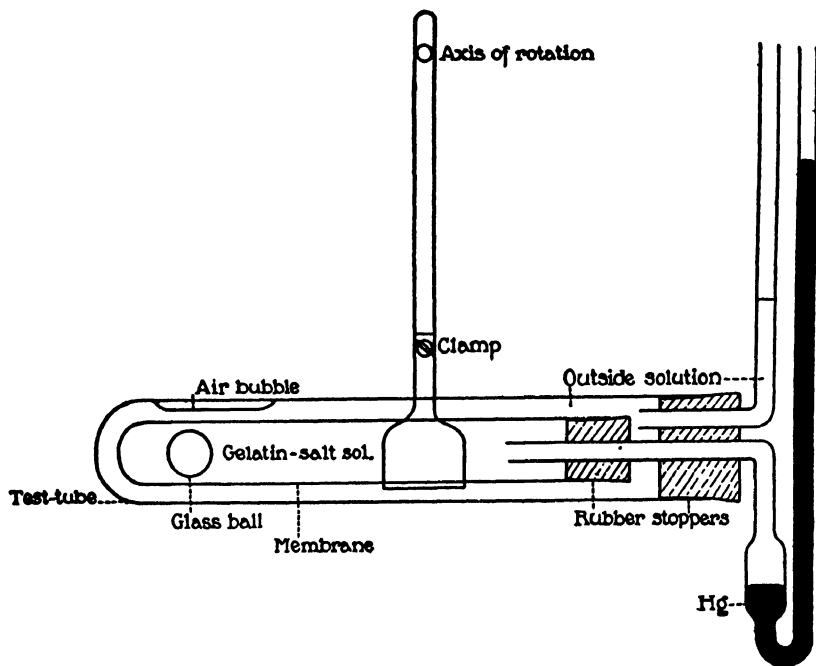
FIG. 2. Osmometer with stirring device.

gm. of water. This obviates also the uncertain correction for the volume occupied by the gelatin which occurs when volume concentrations are used. It was found necessary, in order to shorten the time required for equilibrium, to stir both inside and outside solutions. This was done by placing a large glass ball in the membrane and rocking the entire system, as shown in Fig. 2. The time is further shortened by starting the experiment with equal molalities of electrolyte inside and outside. Control experiments showed that the same value was reached if all the electrolyte was placed outside at first, but that a slightly longer time was required under such conditions. A gelatin solution is therefore prepared so as to contain 10 gm. of

gelatin per 90 gm. of water, and the desired amount of electrolyte. The membrane with its attached manometer and stopper (Fig. 2) is then weighed, filled with the gelatin solution, and again weighed. This gives the total weight of the inside solution and therefore the weight of gelatin. Similarly about 60 cc. of the same molal concentration of electrolyte is measured into a 100 cc. test-tube, and the membrane inserted and held in place by a rubber stopper. The tube is clamped to a rocker arm in the water bath, as shown in Fig. 2, and rocked for 48 hours. At the end of this time, the osmotic pressure is measured with a cathetometer, the manometer tube removed from the membrane, and a curved tube put in its place.
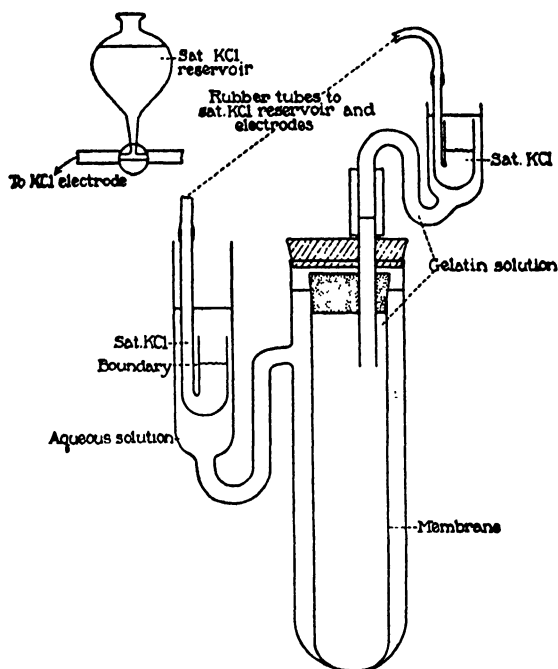
FIG. 3. Membrane arranged for potential measurement.

The outside solution is then poured into a tube with a side arm as shown in Fig. 3, and the membrane inserted in this tube. This tube is shorter than the original outside tube, so that the membrane is pressed against the bottom and sufficient of the inside liquid forced out to fill the bent tube. The arrangement is shown in Fig. 3.

The liquid in the two cups is now connected to two saturated KCl calomel electrodes by means of the bent tubes shown in the figure. In order to obtain constant and reproducible potential measurements it was found necessary to use considerable care in the establishment of the saturated KCl solution–liquid junction. The bent tubes must be quite wide (8 mm.) and are filled with saturated

KCl before inserting in the liquid. They are then washed, and the KCl is removed from about half the depth with a pipette. Liquid from the appropriate solution is then added from a dropper till the tubes are again full, and they are then inserted into the corresponding solution. The junction is then stirred. In this way a wide liquid junction is formed without any sharp boundary, and the potential reading is very constant and reproducible. If narrow tubes are used or if a sharp KCl boundary is formed, the potential drifts and is sensitive to stirring at the boundaries. A type K Leeds and Northrup potentiometer was used for the measurements with a type 2420a galvanometer whose sensitivity had been greatly increased by focusing the image on a scale about 6 m. distant instead of the usual 15 cm. The E.M.F. measured in this way was found to be constant and reproducible to about ±0.1 millivolt.

The Zn^{++} and Cl^- concentration cell measurements and electrode potentials between the inside and outside solutions were made by means of the electrode vessels shown in Fig. 4. The electrode vessels were filled with the appropriate solution and suspended in the water bath over the edge of a cup containing the
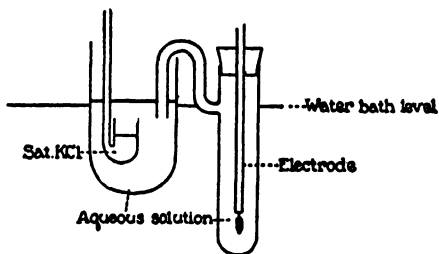
FIG. 4. Arrangement of electrodes for concentration cell measurements.

same solution. Connection with the calomel electrodes was then made in the same way as for the membrane potentials. In using the Zn electrodes it is necessary in order to obtain constant readings to completely fill the electrode vessel and in the case of aqueous solution to slightly acidify the solution.

Owing to the danger of contamination from KCl the analytical data were determined from a separate experiment in which the P.D. was not measured. For this purpose the membrane and its contents were removed and weighed (in order to determine the final concentration of gelatin), and a weighed sample of both inside and outside solution analyzed gravimetrically for Cl. The gelatin solution, after the addition of $AgNO_3$ and 1/3 of its volume of strong nitric acid, is boiled in order to effect complete precipitation. Since in these experiments only pure chlorides were used, the cation concentration can be calculated from the Cl determination. In the case of $ZnCl_2$ a small amount of HCl was added to bring the solution to pH 4.7. The amount of Cl added in this way was corrected for in determining the Zn. From the various weights and the Cl analysis it is therefore possible to calculate the molality of both ions in the outside solution and in the gelatin.

Calculations of the Combined Ions from the Membrane Potential and the Analytical Results.

According to Donnan[6] the membrane potential at equilibrium is equal to $\dfrac{RT}{F} \, ln \, \dfrac{\alpha_o}{\alpha_i}$, where α_o is the activity of any diffusible ion outside the membrane, and α_1 is the activity of the same ion inside. Also, $\alpha_o = \gamma_o M_o$ and $\alpha_i = \gamma_i M_i$, where γ = the activity coefficient and M is the molality of the uncombined ion. A change in α may evidently be due, therefore, either to a change in the activity coefficient γ or to a change in the molality, and at present there seems no way to distinguish with certainty between these alternatives.

According to Lewis and Randall,[7] however, the value of the activity coefficient of any ion in a mixture of strong electrolytes depends only on the "ionic strength" of the solution. A change in the activity due to a change in the activity coefficient should therefore affect the activity of all ions. In the case of proteins, however, this is not true, the activity of some ions (as H^+) being greatly affected by the addition of the protein, while others (as Cl) are only slightly affected. It will be assumed, therefore, in this paper that any change in the activity of an ion on the addition of a protein is due to a change in the concentration of the ion and not to any effect on the activity coefficient. Or, in other words, it is assumed that the protein does not change the "ionic strength" of the solution. In all the experiments reported in this paper the total salt concentration on the two sides of the membrane is very nearly the same, so that γ_o may be considered equal to γ_i, and the formula may therefore be written

$$\text{E.M.F.} = \frac{RT}{F} \, ln \, \frac{M_o}{M_i}.$$

(This, however, is not the case with HCl, since here the total acid concentration inside may be much greater than that outside, and it is necessary to use different activity coefficients; *cf.* Cohn and Berggren.[8])

[6] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572; *Chem. Rev.*, 1924–25, i, 73.

[7] Lewis, G. N., and Randall, M., Thermodynamics, New York and London, 1st edition, 1923.

[8] Cohn, E. J., and Berggren, R. E. L., *J. Gen. Physiol.*, 1924–25, vii, 57.

Let
$$r = \frac{M_o}{M_i},$$

then
$$\ln r = \frac{\text{E.M.F.}}{\dfrac{RT}{F}}$$

or
$$\log r = 0.4343 \frac{\text{E.M.F.}}{\dfrac{RT}{F}}.^{[9]}$$

The ion activity ratios may therefore be calculated directly from the membrane potentials. The actual concentration of the combined ions, however, cannot be calculated without the analytical data. If the ratio of the activities is known from the membrane potential as shown above, and also the total concentration of the ions on both sides of the membrane, then the concentration of combined ions, M_c, is given by the equation

$$M_c = M_i - \frac{\gamma_o M_o}{\gamma_i r}$$

or, if
$$\gamma_o = \gamma_i$$

$$M_c = M_i - \frac{M_o}{r}$$

where M_i is the total molal concentration of the ion inside and M_o is the total molal concentration of the ion outside the membrane.

[9] In using this formula it avoids confusion as to the sign of the potential, if it is remembered that all ions which have the same sign as the calomel electrode in the gelatin (referred to the outside calomel electrode), will have a greater concentration outside the membrane; while all oppositely charged ions will be more concentrated inside the membrane. All electrode potentials will therefore show (between the electrodes) the opposite sign to that of the membrane potential. This calculation is essentially the same as the calculation of combined ion concentration from concentration cells.

Comparison of Electrode and Membrane Potentials and of the Concentration of Combined Ions from Concentration Cell and Membrane Potential Measurements.

The membrane potentials and the potentials obtained with the various electrodes between the inside and outside solutions are given in Table I. The agreement between these figures shows that the system was in equilibrium and confirms Loeb's results that such a system conforms to Donnan's theory with considerable accuracy.

TABLE I.

Comparison of Electrode and Membrane Potentials.

Electrolyte.	ZnCl₂	KCl			HCl
Approximate total molality................	0.10	0.05	0.25	1.0	0.06
Membrane P.D., millivolts,					
Hg HgCl sat. KCl | MeCl | membrane					
| H₂O |	1.14				
|MeCl|sat. KCl HgCl Hg...........	1.24	−0.90	−0.40	−0.08	22.2
|gel |	1.50				
Zn electrode P.D., millivolts,					
Zn |ZnCl₂| sat. KCl| ZnCl₂| Zn...........	−1.50±0.02				
|H₂O | | gel |					
Ag-AgCl P.D., millivolts,					
Ag AgCl| MeCl |sat. KCl|sat.|MeCl|AgCl Ag..	−1.12	0.74	0.33	0.05	
| H₂O | | gel |					
H₂ electrode P.D., millivolts,					
PtH₂|HCl|sat. KCl|HCl|H₂Pt...........					−23.0
|H₂O| | gel |					

A comparison of the combined ions from these measurements is shown in Table II. The agreement is again within a few per cent, except in the case of chloride in high concentration. In this case the figure from the membrane potential depends on a very small difference between two large numbers, so that an error of 1 per cent in analysis may amount to 50 per cent in the figure for combined chloride. All the ions show some combination although in the case of lithium the amount is close to the limit of accuracy of the measurement. It may also be noted that in no case are the amounts of anion and cation

combined equivalent. With $ZnCl_2$, for instance, less than 1 mol Cl is combined per mol of Zn. It is not possible to draw any quantitative conclusion from a comparison of the different ion values since, except in the case of H, they are undoubtedly not maximum values but would increase with increasing concentration of the electrolyte.

TABLE II.

Comparison of Values for Combined Ions by Different Methods.
10 Per Cent Gelatin.

Method.	Electrolyte.	Ion.	Total concentration of ion.	Concentration of combined ion.	Per cent total concentration combined.	Millimols combined per gm. gel.
Concentration cell (37°C.)...............	$ZnCl_2$	Zn	0.010	0.0063	63	0.063
" " (25°C.)...............	"	"	0.011	0.0068	62	0.068
Membrane P.D........................	"	"	0.0126	0.0066	52	0.066
Concentration cell (37°C.)...............	"	"	0.10	0.028	28	0.28
Membrane P.D........................	"	"	0.112	0.027	24	0.27
Concentration cell (37°C.)...............	LiCl	Cl	0.10	0.0068	6.8	0.068
Membrane P.D..................... {	"	"	0.1049	0.008	7.6	0.08
	"	"	0.106	0.0096	9.1	0.09
" " .... {	"	Li	0.1049	0.0035	3.3	0.035
	"	"	0.106	0.0050	4.8	0.05
Concentration cell (33°C.[*]).............	HCl	H				0.92
Membrane P.D. (4.67 per cent gel)...... {			0.06	0.0428	70	0.916
			0.06	0.0427		0.917

[*] Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 739.

SUMMARY.

A method is described for measuring membrane potentials of gelatin-salt solutions, and it is pointed out that such measurements, together with the analysis of the solutions, allow the calculation of the concentration of ions combined with the protein.

The values for the combined ions obtained in this way for $ZnCl_2$, KCl, LiCl, and HCl agree quite well with those obtained by direct concentration cell measurements.

THE RESISTANCE OF LIVING ORGANISMS TO DIGESTION BY PEPSIN OR TRYPSIN.

By JOHN H. NORTHROP.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The fact that living organisms are not digested by pepsin or trypsin, whereas dead organisms are rapidly digested, has attracted the attention of biologists and physiologists for many years. The early work was centered chiefly about the defense of the digestive tract, but it was soon recognized that all living matter was resistant to these enzymes. The problem is evidently closely connected with the resistance of the living cell to autolysis by the enzymes already in the cell. It differs from the latter in that by using concentrated solutions of the proteolytic enzymes the digestion of dead cells may be made very rapid in comparison to autolysis. There is a much more striking difference, therefore, in the behavior of dead and living cells when exposed to trypsin or pepsin than in the autolysis of dead and living cells. Only the resistance to pepsin and trypsin is considered in this paper.

Fermi[1] was able to show in a series of careful and ingenious researches that the theory of a protective mucus or epithelium of Claude Bernard, Pavy's theory of the alkalinity of the tissues, Gaglio's theory of the absorption of the enzymes, and the antienzyme theory of Wieland were all insufficient to fully account for the results. There is no doubt that the alkalinity of the blood and the presence of antienzymes have an effect on the process, but, as Fermi points out, they are insufficient to account for the complete absence of digestion of living cells. Fermi concluded that the configuration of the protein molecule in the living cell was different from that after the death of the cell, and that the "living molecule" could not be attacked by the enzyme. This theory is not very different from Hunter's "living

[1] Fermi, C., *Centr. Bakt.*, *1. Abt., Orig.*, 1910, lvi, 55.

principle" except that the attribute of "living" is applied specifically to the protein molecule. Fermi considered that the assumption of a mechanism which prevented the enzyme from entering the living cell was untenable, since there are innumerable types of cell membranes, and it is not reasonable to suppose that they are all impermeable to the enzyme while the cell is living and permeable after the death of the cell. It is evident, however, that if there were such a mechanism it would account for the failure of the enzymes to destroy the cell. The work of Osterhout[2] and others has shown that the permeability of the cell is one of the most characteristic attributes of the living cell and that this permeability changes in a remarkable way when the cell is dead or injured, so that it is not at all unlikely that the permeability to enzymes is also greatly different in the living and the dead cell. It is known also that pepsin and trypsin, at least, are secreted in an inactive form. The present experiments were undertaken, therefore, to determine whether or not pepsin and trypsin actually did enter the living as well as the dead cell. It was found in every case that as long as the cell was alive, no detectable quantity of enzyme was taken up; whereas when the cell died the enzyme was rapidly removed from solution and concentrated in the cell.

Effect of Trypsin on Living and Dead Organisms.

The results of several experiments in which living and dead organisms were exposed to the action of a powerful dialyzed trypsin solution are shown in Table I. They confirm the results of Fermi and other workers, in showing that the living cell is not attacked whereas organisms which have been killed by heat or mechanical injury are rapidly digested. The results were more striking than the figures show, since in some cases the dead organism was almost completely disintegrated although the formol titration increase was small. It may be added that the animals lived just as long in the active enzyme solution as in the inactivated enzyme. The experiment also shows that the slow digestion due to autolysis is negligible compared to that due to the trypsin.

[2] Osterhout, W. J. V., Injury, recovery, and death, in relation to conductivity and permeability, Monographs on experimental biology, Philadelphia and London, 1922.

The digestion of the organisms killed by heat could be readily accounted for by assuming a change in the chemical nature of the proteins or by the destruction of the antienzyme. These objections, however, do not apply to the digestion of the organism when killed simply by mechanical injury. It is difficult to imagine that this would cause a change in the chemical nature of the protein, unless Fermi's "living molecule" is assumed. The antienzyme is present and in the case of earthworms its action is marked, yet, owing to the large excess of enzyme, the tissue digests.

TABLE I.

Effect of Trypsin on Living and Dead Organisms.

Organisms added to concentrated dialyzed trypsin and left at 20°C. for 24 hours. Increase in formol titration and visible digestion determined.

	Appearance and increase of formol titration, 0.1 N NaOH per cc. solution after 24 hours.					
	Living organisms.		Killed by cutting.		Killed by boiling 10 min.	
	Active trypsin.	Inactive trypsin.	Active trypsin.	Inactive trypsin.	Active trypsin.	Inactive trypsin.
Earthworm*.....	0.8, alive.	0.9, alive.	3.50, nearly all digested.	1.0	3.50	—
Euglena.........	—, "				Cells dissolved.	
Yeast..........	—, "				0.75, cells dissolved.	
Fundulus........	0 1, "	0.1, "	0.3, partially digested.	—	0.8, partially digested.	0.10
Meal worm†....	0.1, "	—, "	2.0	0.8	0.4	—
Goldfish‡........	—, "	—, "	0.20	—	0.35	—

* *Lumbricus terrestris.*   † *Tenebrio molitor.*   ‡ *Carassius auratus.*

Permeability of Dead and Living Tissue to Pepsin and Trypsin.

In order to see whether the enzyme can penetrate into the organisms the experiments were repeated with the modification that the organisms were placed in a small volume of the enzyme solution. This is necessary since otherwise the change in concentration of the supernatant enzyme solution, on removal of a small quantity of trypsin, would be too small to measure. The concentration of the enzyme in the supernatant solution was then determined at intervals by the vis-

cosity method described by Hussey and the writer.[3]   Since the volume cf the enzyme solution is known, the total amount of enzyme removed can be calculated.   In every case the dead organism removed large quantities of the enzyme from solution, while the living organism removed little or none (Table II).   Pepsin and trypsin therefore cannot enter the living tissue.   It might be objected that even though it can not enter, the enzyme should attack the surface of the cell.   There is considerable reason to suppose that the surface of the cell is not protein in nature, but even though hydrolysis did take place at the surface,

TABLE II.

Removal of Trypsin from Solution by Living and Dead Organisms.

Material washed with dilute trypsin solution and placed in an equal volume of dilute trypsin at 0°C. for 24 hours.   Supernatant solution tested for trypsin.

	Total units trypsin removed from solution.				
	Living.	Killed by cutting.	Killed by boiling.	Killed by HCl.	Killed by 50 per cent alcohol.
B. coli..........	0		4.0		
Earthworm......	1.0	10.0	14.0		
Euglena........	0		6.0		
Meal worm.*....	4.0	6.0	7.0		
Goldfish........	0		4.0		
	Pepsin.				
Earthworm......	0	6.0	8.0	10.0	4.0

* Worms injured, some motion but do not recover on removal from solution.

it would be so slow compared to the rate when the enzyme was distributed throughout the cell that it could not be detected experimentally. This may be seen from the following approximate calculation.   Suppose a block of protein, $1\mu = 1000\ \mu\mu$ cube, consists of protein molecules which are $10\mu\mu$ cube.   The block will contain $10^9\ \mu\mu^3$ or $\dfrac{10^9}{10^3} = 10^6$ molecules.   The surface layer of the block will contain only $\dfrac{6 \times 10^6}{6 \times 10^2} = 10^4$ molecules.   The rate of digestion of the block from the

[3] Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, 1922–23, v, 353.

surface only will be, therefore, $\frac{1}{100}$ of the rate if the whole block were attacked. The difference will be still greater owing to the actual concentration of enzyme inside the particle, and becomes rapidly greater as the cell becomes larger.

It may be shown by the following experiment that the enzyme is really taken up in an active form and is not merely inactivated or destroyed. Pieces of earthworms which have been killed by mechanical injury or heat are placed in a strong trypsin solution for 1 hour. The trypsin is rapidly removed from solution but in this time no noticeable digestion occurs. The tissue is then removed from the enzyme solution, washed with water, and placed in a small volume of water. After 24 hours at 20°C. the tissue is practically completely digested, while control pieces treated in the same way with inactivated trypsin are unchanged.

If the lack of hydrolysis of living cells were really due to the protective action of the membrane, it should be possible to show that digestion occurs if the enzyme is actually injected into the cell. This experiment has been performed with *Amœba*, using Chambers' microinjection technique.[4] The following are typical experiments.

1. Several *Amœba* placed in active and in inactivated 5 per cent dialyzed trypsin remain normal more than 6 hours.

2. *Amœba in active trypsin.* Entire cell sucked into capillary pipette and then blown out. Mass of granules embedded in jelly-like protoplasmic mass. This mass is rapidly digested and after 4 to 5 minutes only the granules are left.

A volume of active trypsin equivalent to about 1/3 to 1/4 the volume of the cell was injected. Rapid streaming commences, the injected solution collects into a spherical blister containing granules. The cell contracts in an "attempt" to pinch off this blister. The streaming stops and, although the blister may be pinched off, the cell becomes motionless and disintegrates in the course of a few hours. The membrane of the blister is not attacked and the spherical shape may be retained for some time.

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[4] Chambers, R., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 66; *J. Gen. Physiol.*, 1922–23, v, 189.

Injection of the same trypsin solution inactivated by heat results merely in the rapid pinching off of the blister, as described by Chambers in the case of oil drops.

Further confirmation of the idea that the resistance of living organisms to external enzymes is due to the cell membrane may be found in the fact that the injection of lipase into living organisms causes rapid hydrolysis of the extracellular fat.[5]

Mechanism of the Concentration of the Enzyme in Dead Tissue.

It has long been known that coagulated proteins remove pepsin or trypsin from the surrounding solution. It was found by the writer that the relative concentration of the enzyme inside and outside of such protein particles was the same as that of other ions whose distribution was determined by the Donnan equilibrium. It seems necessary to assume, therefore, that the enzymes are ions and that their distribution is determined in the same way as that of other ions. There seems every reason to suppose that a piece of dead tissue acts in the same way as a particle of denatured protein and that the mechanism regulating the distribution is here also the Donnan effect.

SUMMARY.

1. Pepsin and trypsin are quickly removed from solution by dead organisms. They are not able to penetrate into living organisms.

2. Trypsin injected into a living *Amœba* results in the death and disintegration of the cell.

[5] *Cf.* Wells, H. G., Chemical pathology, Philadelphia and London, 3rd edition, 1918, 385.

MECHANISM OF THE ACCUMULATION OF DYE IN NITELLA ON THE BASIS OF THE ENTRANCE OF THE DYE AS UNDISSOCIATED MOLECULES.

BY MARIAN IRWIN.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

I.

INTRODUCTION.

The mechanism of the accumulation of the basic dye, brilliant cresyl blue, in the sap of *Nitella* has been discussed by the writer[1] on the basis of experiments made with different concentrations of the dye at one pH value. More recently a preliminary report[2] was made by the writer on experiments[3] with one concentration of the dye at different pH values. A fuller account of these experiments is given in the present paper. In order to understand the mechanism it is necessary to analyze the data for (1) the rate of penetration of the dye into the cell sap, and for (2) the final equilibrium.

II.

Methods.

The living cells[4] of *Nitella* were placed at 25°C. $\pm$ 0.5° in 2×10^{-5} M dye solutions (brilliant cresyl blue) at different pH values, from pH

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[1] Irwin, M., *J. Gen. Physiol.*, 1925–26, viii, 147.

[2] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 251.

[3] Experiments such as these were made by the writer on *Nitella* found in Woods Hole, Massachusetts, with brilliant cresyl blue made by the National Aniline Chemical Company, but at that time the dissociation constant of the dye was not known, so that the analysis of the data was not complete. Since it is no longer possible to obtain the *Nitella* at Woods Hole, the experiments were repeated with *Nitella* found at Cambridge, and with the dye made by Grübler, the dissociation constant of which was found by the writer as will be described later. Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 727.

[4] For details of technique see the writer's paper referred to in Foot-note 1. The *Nitella* used was obtained from Cambridge, Massachusetts.

6.1 to pH 9.3 (M/150 phosphates or borates as buffers).[5]   The concentration of the dye was kept constant throughout each experiment. At definite intervals the cells were removed from the solutions.   The end of each cell was then cut and the sap was squeezed out upon a glass slide.   The sap was then drawn up into a capillary tube, the color of which was matched with that of the capillary tube containing the standard dye solution.

<div align="center">III.</div>

<div align="center">*Analysis of the Rate of Penetration.*</div>

When the concentrations of the dye in the sap were thus determined at definite intervals, it was found that the greater the pH value of the external solution, the higher was the rate of penetration.   The maximum was reached at about pH 9.3, where further increase in pH value of the external solution brought about no appreciable increase in the rate of accumulation.   We may assume that the dye behaves as a weak base and that the dye ions cannot enter but that the dye penetrates only in the form of undissociated molecules of the free base which for convenience will be referred to as DOH.   In that case, the rate of penetration at the start should be proportional to the concentration of DOH in the external solution.   With a constant concentration of the dye the concentration of DOH will depend on the pH value.   If this assumption is correct, we can calculate the concentration of DOH, expressed as per cent of the total dye present from the rates of penetration into *Nitella*, and this should agree with the values obtained by other methods such as that of determining the distribution[6] of

[5] The readings made at pH 6.1 are rather uncertain because at this pH value the cellulose wall becomes deeply stained, and it is difficult to avoid contamination of the sap from the dye adhering to the cell wall at the cut end.   The readings made with pH values lower than this cannot be used since the lower the pH value the more rapid is the staining of the cellulose wall and the greater the chance of contamination.

[6] Pure chloroform was added to 1.4×10^{-5} M dye solution previously saturated with chloroform at different pH values (M/150 phosphates or borates) at 24°C. $\pm$ 1°, and the determination of the amount of dye taken up by chloroform at equilibrium was made colorimetrically.   The color of the dye in water is blue at the pH values used, but in chloroform it is pink (when the dye comes out again into water it is blue).   In order to make the colorimetric determinations accurate,

the dye between chloroform and water, which was employed by the writer.

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it was necessary to use different volumes of chloroform and the dye solutions at different pH values, so that at equilibrium the concentration of the dye will be reduced to about 0.000007 M. Such mixtures were shaken vigorously in a separatory funnel, and after equilibrium was established the chloroform was allowed to separate and was then drawn off. The aqueous solution was then collected in a test-tube, and tightly stoppered at once. Extreme care must be taken to avoid the slightest evaporation of the chloroform in the funnel or in the test-tube, or else the aqueous solution will at once become more concentrated by taking up the dye left by the evaporated chloroform. The color of the tube containing the aqueous solution was matched with that of tubes of the same diameter containing standard dye solutions. Since the volume of the aqueous solution, the volume of the chloroform, and the concentration of the dye in the aqueous solution at start and at equilibrium were known, the concentration of dye in the chloroform at equilibrium could be readily calculated. When this was done, it was found that the relative amount (distribution coefficient) of dye taken up by the chloroform increased with increase in the pH value of the aqueous solution, until a maximum was reached at about pH 9.3, when further increase in the pH value brought about no appreciable increase in the taking up of the dye. This was not due to the saturation of the dye in the chloroform, because more dye was taken up on raising the concentration of the dye in the aqueous solution. At this pH value it may be assumed that 88 per cent of the dye in the aqueous solution is in the form of undissociated DOH. On this basis, it is possible to calculate the value of the constant at the pH value where 100 per cent of the dye is undissociated DOH, by the equation $\dfrac{C_1}{C_2\,(1-\alpha)} = K$, in which C_1 is the concentration of undissociated DOH in chloroform, C_2 is the concentration of the total dye in water, K is the constant, and α is the molar fraction of the dye dissociated. By substituting 0.88 for α and the observed values of C_1 and C_2 at pH 9.3, the value of K was determined where all of the dye in the aqueous solution was in the form of undissociated DOH, and was found to be 780.

There seems to be no association of the dye in the chloroform because dilution of the aqueous dye solution does not change the value of the constant.

The degree of dissociation of the dye may now be calculated at different pH values by using the above equation.

The percentage of undissociated DOH calculated in this manner for various pH values of the aqueous solution from pH 5.3 to 9.3 is shown by the symbol $\times$ in Fig. 1. From this curve the dissociation constant of DOH is determined graphically to be $10^{-5.6}$. Cf. Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 2nd edition, 1922, 44, 46, in which pOH is substituted for pH and OH for H.

This method was checked by another in which the chloroform containing the dye was removed from the aqueous solution and placed in 2 cc. of water after

When the rates[7] (the reciprocal of time taken for the concentration of the dye in the sap to reach 3.45×10^{-5} M) for different pH values of the external dye solutions are calculated, it is found that a maximum is reached at about pH 9.3, as already stated. This is regarded as indicating that the percentage of undissociated DOH has nearly reached its maximum value. We assume[8] that this is 88 per cent of the total dye and the per cent of undissociated DOH at different pH values is calculated on this basis by assuming that the rate of penetration is directly proportional to the concentration of DOH. When such values are plotted against the external pH values, the curve agrees closely with that obtained by the experiments on the distribution of the dye between chloroform and water, as shown by the symbols $\times$ and $\bigcirc$ in Fig. 1. The theoretical curve, calculated from the dissociation constant of the dye,[9] $K = 10^{-5.6}$ follows these two curves,

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which the chloroform was driven off by a current of air. After complete evaporation of the chloroform the solution was diluted to a point at which a good colorimetric determination could be made. The results thus obtained agreed closely with those described above.

Distribution of the dye between benzene and water was determined at different pH values. The constant, K, of the partition coefficient was found to be lower than for chloroform so that for very high pH values it was more satisfactory but for pH values below 8 it was so unsatisfactory that the results obtained by this method were not seriously considered. The dissociation constant was found to be about $10^{-5.2}$.

[7] The rates taken with 0.000014 M dye in the sap gave the same type of curve. The rates were taken at a low concentration to avoid the possibility of error from having the pH values of the sap affected by the dye. The results seem to indicate that so long as the concentration of the dye in the sap does not go above 0.0000345 M such errors as the above described are avoided. If we compare the amounts of dye taken up by the sap at different pH values near the start of the experiment (at 3 minutes) we get the same type of curve.

[8] This value was chosen as producing the best agreement among the curves shown in Fig. 1.

[9] The equation used for the calculation of α, the fraction of the dye dissociated is:

$$\alpha = \frac{1}{1 + \dfrac{OH}{K}}$$

in which K is $10^{-5.6}$. In a previous paper (Foot-note 1) this was stated as K = $10^{-6.4}$. Since, however, the dissociation constant could not be accurately determined at that time, the calculations were not published in detail. This has been remedied by improved technique.

as shown by the curve as drawn in Fig. 1 until about pH 7, below which the chloroform curve becomes lower than the calculated (unfortunately this difference is not well marked in the figure because the scale
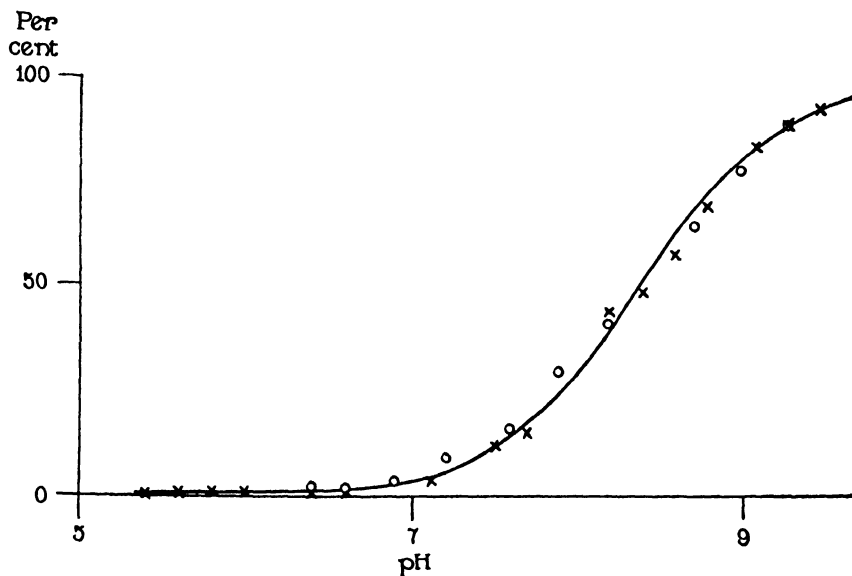
FIG. 1. Curve showing the percentage of undissociated molecules of brilliant cresyl blue at different pH values. The ordinates represent the percentage and the abscissæ represent the pH values. Symbol ○ represents the data from rates of penetration of the dye into *Nitella*. Symbol × represents the data from the distribution of the dye between chloroform and water. The curve as drawn represents the calculation made from the equation, $\alpha = \dfrac{1}{1+\dfrac{OH}{K}}$, when $K = 10^{-5.6}$.

for plotting is very small at these pH values). This difference may be due either to experimental errors or to the dissociation of a second salt-forming group in the dye molecule. If we put $K_1 = 10^{-5.6}$ and $K_2 = 10^{-8}$ the theoretical[10] curve agrees more closely with the chloro-

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[10] The equation used for the calculation of ρ, the fraction of the dye undissociated is $\rho = \dfrac{1}{1 + \dfrac{K_1}{OH} + \dfrac{K_1 K_2}{(OH)^2}}$ in which $K_1 = 10^{-5.6}$, and $K_2 = 10^{-8}$.

form curve, but since the chances for experimental errors are rather great at these lower pH values, the writer does not wish to make a definite statement as to the nature of this difference until an opportunity presents itself to determine the dissociation constants more accurately.

These results seem to indicate that the dye enters only in the form of DOH, and that the rate of penetration is directly proportional to the concentration of DOH in the external solution, provided the conditions in the cell are kept constant.   Changes in the condition of the cell sap, for example, can alter the rates of penetration, as has already been shown,[11] though none of the experiments are very reliable since the cells might have been injured with the changes in the pH values of the sap.   The sap of *Nitella* is buffered, according to Hoagland and Davis,[12] so that, in all probability, the presence of 3.45×10^{-5} M dye in the sap brings about no change in the pH value of the sap.   Even if the pH values are increased by the presence of this amount of dye in the sap, the relative rates will not change so long as the pH value of the sap is changed to the same extent for all external pH values.

[11] McCutcheon and Lucke, and later the writer, have found that an increase in the pH value of the sap brought about a decrease in the rate of penetration (cells may be injured). Recently the writer has found that when acetic acid penetrates the living cells of *Nitella* until the pH of the sap is changed from pH 5.6 to 5, an increase in the rate of penetration of the dye takes place.   This experiment is unreliable because there is formed a white precipitate in the sap (in all probability the protein in the sap has reached its isoelectric point), and the cells may be injured.   The fact that after a brief exposure to NH_3 the rate may be decreased before the pH value of the sap is increased may be due to the fact that the NH_3 is at that time confined to the protoplasm and the outer portion of the sap, where it could affect the rate by locally raising the pH value without, however, affecting the pH value of the sap as a whole when squeezed out on the slide, or due to the fact that in correspondence with the conditions in the sap, there is present NH_3 (without the change in the pH value) in the parts of the cell other than the vacuole (McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501. Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 235.)

[12] Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1922–23, v, 629.

IV.

Analysis of the Equilibrium.

The experiments described in Section II may be regarded as indicating that the dye enters the cell only in the form of DOH. It is desirable to inquire whether the analysis of the equilibria will support this interpretation.

At pH 6.4, 6.6, and 6.9, the absorption of dye reaches an equilibrium, but at higher pH values of the external dye solutions the cells die before the equilibrium was attained. The equilibrium values thus obtained increase as the pH values of the external solutions rise.

If the dye penetrates as DOH we shall expect that at equilibrium the internal and external concentrations of DOH will be the same. Thus when the external dye solution is 2×10^{-5} M and the external pH is 6.9, the concentration of DOH in the external solution may be taken as 3.16 per cent of 0.00002 M (since according to the theoretical curve, 3.16 per cent of the dye is in the form of DOH at pH 6.9). Hence we have 6.31×10^{-7} M DOH in the external solution and in the sap at equilibrium. In the sap the pH value may be taken as 5.6, at which value DOH forms 0.16 per cent of the total dye (according to the theoretical curve). Hence when DOH enters the sap it must dissociate, forming a sufficient number of ions to constitute 99.84 per cent of the total dye inside. If we assume that these ions cannot escape from the cell vacuole, then the total dye, x, inside will be

$$x = \frac{100}{0.16} \times 0.000000631 \text{ M} = 0.000395 \text{ M}$$

whereas we actually find 0.00014 M by observation.[13] Table I shows the corresponding values calculated for the pH values 6.4 and 6.6.

At all of the pH values the calculated values are higher than the observed.

If the above assumption is correct, then it should be possible to treat in the same manner the previous determinations[1] of the concentrations of the dye in the sap at equilibrium with different concentrations of external dye solutions at one pH value. When such

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[13] Cf. Osterhout, W. J. V., and Dorcas, M. J., J. Gen. Physiol., 1925–26, ix, 255.

TABLE I.

From the percentage dissociation of brilliant cresyl blue calculated when $K = 10^{-5.6}$ the values of the total dye (D^+ ions and DOH) in the sap of *Nitella* at equilibrium are calculated at different pH values of the external dye solutions at 25°C. The concentration of the external dye solution is 2 M $\times 10^{-5}$, and the pH value of the sap is 5.6, at which pH value 0.16 per cent of the total concentration of the dye is undissociated. The calculations for Tables I and II were made with a 20 inch slide rule.

pH of the external dye solution.	Concentration of undissociated molecules in the external solution.	Concentration of undissociated molecules in the sap and in the external solution.	Observed values of the total concentration of the dye in the sap.	Calculated values of the total concentration of the dye in the sap.
	per cent	M $\times 10^{-5}$	M $\times 10^{-5}$	M $\times 10^{-5}$
6.4	1	0.03	6	12.5
6.6	1.6	0.036	9	20.0
6.9	3.16	0.076	14	39.5

TABLE II.

From the percentage dissociation of brilliant cresyl blue calculated when $K = 10^{-5.6}$ the values of the total dye (D^+ ions and DOH) in the sap of *Nitella* are calculated for different concentrations of external dye solutions at 25°C. The pH of the sap is 5.6, at which pH 0.16 per cent of the total concentration of the dye is undissociated. At the pH value of the external dye solution (pH 6.9) 3.16 per cent of the dye is undissociated.

Concentration of external dye solution.	Observed values of total concentration of dye in the sap.	Calculated values of the total concentration of dye in the sap.
M $\times 10^{-5}$	M $\times 10^{-5}$	M $\times 10^{-5}$
0.40	2.8	7.9
0.65	4.4	12.8
1.00	6.9	19.8
1.30	9.0	25.7
1.5	11.0	29.6
1.7	12.4	33.5
2.0	14.1	39.5
2.6	27.5	51.3
3.1	32.0	61.2
4.1	46.5	81.0

calculations are made, it is found, as shown in Table II, that the values of the total dye in the sap are higher than the observed. The fact that the observed values are lower than the calculated cannot be

wholly due to the increase in the pH value of the sap brought about by the presence of the dye in the sap, because, if this were the case, the extent of the lowering of the concentration of the dye in the sap should increase proportionally as the concentration of the dye in the sap is increased, but this does not seem to be the case. Such a lowering may be due to the fact that the dye is not so soluble in the sap as it is in the external solution or that the dissociation constant of the dye is not the same in the sap as it is in the external solution. Unfortunately there is not sufficient quantity of sap available to determine this point.

When the concentration of the dye has reached about 0.00014 M in the sap, the disagreement between the observed values and the calculated becomes less. This may be due to the occurrence of secondary changes in the cell, which increase the final concentration of the dye in the sap, as already suggested[1] by the writer.

If the values of the dye in the sap are calculated on the assumption that there are two dissociation constants, by using the values for the undissociated DOH calculated from the equation[10] already described, the discrepancy between the calculated values and the observed values of the dye in the sap is still greater.

Furthermore, if a correction is made for the ionic strength of the sap (about 0.1 M, comprising NaCl and KCl in about equal proportions) the discrepancy becomes still greater.

Let us now see if the values calculated on the basis of the Donnan equilibrium which is based on the entrance of ions will not agree with the observed. According to the Donnan equilibrium the relation $\dfrac{H^+ \text{ inside}}{H^+ \text{ outside}} = \dfrac{D^+ \text{ inside}}{D^+ \text{ outside}}$ must hold if the dye behaves as a monoacid base. When the values of D^+ ions inside are calculated on this basis, it is found that the calculated values are higher than the observed to the same extent as found in the case of the values calculated on the basis of the entrance of the dye as undissociated molecules of DOH when $K = 10^{-5.6}$

If the dye behaves as a diacid base with K_1 equal to K_2, the relation $\dfrac{D^+ \text{ inside}}{D^+ \text{ outside}} = \dfrac{(H^+)^2 \text{ inside}}{(H^+)^2 \text{ outside}}$, in which case the discrepancy between the observed and the calculated values is still greater.

In case K_1 is not equal to K_2 the calculation of the dye in the sap is somewhat complicated.

Whether the dye behaves as a monoacid or diacid base it would not be possible to distinguish from an analysis of the conditions in the sap at equilibrium if the dye enters the cell as undissociated dye base or as ions. We cannot assume that $\dfrac{\text{H}^+ \text{ inside}}{\text{H}^+ \text{ outside}} = \dfrac{\text{D}^+ \text{ inside}}{\text{D}^+ \text{ outside}}$ unless H$^+$ ions are diffusible through the protoplasm, but such is not the case with *Nitella*. The Donnan equilibrium requires that all the diffusible cations should stand in the same relation (inside to outside) as the H ions, but this is not the case. Furthermore the relation of Cl ions should be the reverse of that of the cations, but as a matter of fact the contrary is true. It therefore does not seem probable that the results can be explained on the basis of the Donnan equilibrium. Moreover, in all probability the ions do not enter, as pointed out by Osterhout and Dorcas,[13] because, if the rate of penetration increases with increase in the outside concentration of undissociated molecules the conditions at equilibrium cannot be due to the Donnan effect unless the undissociated molecules penetrate much more rapidly than the ions.

<p style="text-align:center">V.</p>

<p style="text-align:center">DISCUSSION.</p>

The above analysis seems to indicate that the rate of penetration of the dye into living cells of *Nitella* is proportional to the concentration[14] of undissociated molecules of the dye in the external solution, provided the conditions in the cell remain the same for all the external pH values. Since the temperature coefficient between 20° and 25°C.

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[14] The following writers have assumed that a basic dye enters a living cell in the form of undissociated molecules, Overton, E., *Jahrb. wissensch. Bot.*, 1900, xliii, 669. Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507. Robertson, T. B., *J. Biol. Chem.*, 1908, iv, 1. McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501. Brooks, M. M., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 265. Referring to her experiments on the penetration of 2, 6-dibromophenol-indophenol into *Valonia*, Brooks states that "the amount of dye in the sap at equilibrium is proportional to the amount of undissociated dye in the external solution." Without further details, the writer is unable to determine whether in this case the dye enters as undissociated molecules or not.

is about 4.8, the rate cannot be dependent on the simple diffusion of DOH into the cell vacuole. The process may be complicated by a chemical combination of the dye in the protoplasm, or in the membranes, which might be a slower process than diffusion. This idea is supported by the following observation. In the experiments of Osterhout and Dorcas[13] on the penetration of CO_2 into living cells of *Valonia*, the temperature coefficient is very low (that of diffusion), while in the case of the writer's experiments on the penetration of brilliant cresyl blue into *Valonia* the temperature coefficient is very high (that of a chemical reaction). This leads the writer to believe that CO_2 enters the cell vacuole without combining with protoplasmic constituents, while the dye enters into combination. It may be possible that the dye enters by diffusion complicated by some other factors which are unknown to us at present.

In either case, it might well happen that the time curve of penetration of the dye into the cell vacuole would follow the equation for a unimolecular reaction as described.[1]

The treatment of the time curves made in the writer's previous papers on the penetration of the dye into the living cells of *Nitella* will hold on the basis of the present theory.

As to the conditions at equilibrium, the analysis seems to indicate that the final concentration of the dye is governed by the concentration of DOH in the external solution and by the percentage of dissociation of DOH in the sap (provided there are no complications due to other factors), as previously suggested by Osterhout and Dorcas[13] in discussing the penetration of CO_2 into *Valonia*.

In case there is a combination of the dye with a constituent, XA, of the sap, according to the equation DOH + XA $\rightleftharpoons$ DA + XOH, the total dye in the sap would be composed of DOH, D^+ ions, and DA (all of the same color), and the calculations would have to be made accordingly. If DA were slightly soluble or slightly ionized, the concentration of D^+ ions and of DOH would remain the same as if DA were not present, unless the solubility or the pH values are changed by the presence of DA.

It may be added that all that has been said regarding DOH would apply equally well to a tautomer of the dye which acts similarly to DOH.

The solubility of the dye in the sap is also an important factor to be considered in relation to the penetration. It is of interest to mention here that methyl red, even at pH 8 or 9, where the dye is practically in the form of undissociated DOH and can be readily absorbed by the chloroform, cannot enter the cell sap, and it may be that this is due to the fact that methyl red is not very soluble in the sap. Or, it may be possible that there is a specificity in the behavior of the cell toward the undissociated molecules. That not all undissociated molecules enter may be still further shown by the fact that the acid dyes, such as thymol blue, brom thymol blue, phenol red, brom cresol purple, at pH 5.5, where the greater percentage of the total dye is in the form of undissociated HD molecules, do not enter the cell. These indicators are not very soluble in aqueous solution and in chloroform, so that this may be interpreted as being due to the still greater lack of solubility of the dye in the sap and in a lipoid, but a dye, such as acid fuchsin, which is readily soluble in water and slightly soluble in chloroform does not enter the cell. Further investigation is now being undertaken, and in the near future the writer hopes to throw some light on this problem.

The assumption that the ions do not enter appreciably is still further supported by the experiments on other basic dyes, crystal violet, malachite green (nitrate), and tetramethyl diaminophenoxazonium nitrate, neutral red, and methylene blue,[15] the rate of penetration of which depends chiefly on the concentration of undissociated DOH molecules.

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[15] The writer is indebted to Dr. W. A. Jacobs and Dr. M. Heidelberger of this Institute for their kindness in supplying her with the first three dyes in highly purified form.

The methylene blue was purified by repeated recrystallization and extraction with chloroform. This dye does not appreciably enter the living cells of *Nitella* and *Valonia* at pH 5.4. In case the ions enter they enter so slowly that it is difficult to determine whether or not the presence of the dye in the sap is due to the contamination of the sap from the stained cell wall, or to an injury. This result is contrary to the results obtained by Brooks[14] on the penetration of the dye into *Valonia*. Methylene blue is not a good dye to use for this purpose because it is very difficult to separate it in pure form from other dyes which behave as weaker bases and which are mixed with it in great quantity, so that we cannot tell at higher pH values whether the dye which enters is methylene blue or other dyes which are not so strongly dissociated.

The mechanism of the penetration of the dye into living cells of *Nitella* represents by no means a simple process, and though the results tend to confirm more and more the assumption discussed in this paper, the writer disclaims any intention of attempting a complete explanation at present.

SUMMARY.

The rate of penetration of brilliant cresyl blue into the living cells of *Nitella* indicates that the dye enters only in the form of the undissociated molecule. At equilibrium the total concentration of the dye in the sap is proportional to the concentration of the free base in the outside solution.

A STUDY OF HEMOLYTIC STREPTOCOCCI IN ACUTE RHEUMATIC FEVER, WITH AN ANALYSIS OF THE ANTIGENIC RELATIONSHIPS EXISTING AMONG CERTAIN STRAINS.

BY CHRISTOPHER H. ANDREWES, M.D., CLIFFORD L. DERICK, M.D., AND HOMER F. SWIFT, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

INTRODUCTION.

The part played by streptococci in acute rheumatic fever has long been a subject for discussion, with the chief attention directed towards *Streptococcus viridans.* Hemolytic strains have, however, been isolated at times from the blood and other sources in patients with this disease. It has long been noticed that many patients give a history of a sore throat shortly before the onset of their acute arthritis. Of 122 admissions for rheumatic fever to the Hospital of The Rockefeller Institute during the last few years, a history of a recent acute sore throat was obtained from 62 patients—or, roughly, 50 per cent. Since one of the commonest organisms associated with an acute tonsillitis or pharyngitis is a hemolytic streptococcus, it seemed of interest to seek for, and, if found, to study these organisms in the throats of patients early in the course of rheumatic fever. A difficulty here arose: by the time the patients with arthritis enter the hospital most of them have more or less completely recovered from their throat infection and show at most slight redness of the tonsils or pharynx. During the past 5 years at this Hospital, only 5 rheumatic fever patients have been seen in whom the throat infection was really active at the time of admission. Throat cultures were made from 4 patients within 10 days of the onset of their acute throat infection: in two, hemolytic streptococci were the predominating organisms; in the third, there were only a few hemolytic streptococcus colonies; in the fourth, there was an almost pure culture of *Streptococcus viridans.*

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It has been shown that in several diseases associated with hemolytic streptococci these have special serological characters. Tunnicliff (1), Dochez and Bliss (2), Gordon (3), and others found that streptococci from patients with scarlet fever fall into a definite serological group. Tunnicliff (4) and Birkhaug (5) have demonstrated a similar relationship among erysipelas strains. Dochez, Avery, and Lancefield (6), also, found 4 well defined groups in a pneumonia epidemic in Texas. We accordingly endeavoured to ascertain if the strains isolated from patients with rheumatic fever were serologically related. The importance of isolating such strains as early as possible in the disease is evident especially since Eagles (7) has presented evidence to show that the streptococci from the fauces of patients with scarlet fever may, during the early weeks of the disease, change in their power to be agglutinated by a single immune serum. No attempt will be made in this paper to discuss what part, if any, is played by hemolytic streptococci in the etiology of rheumatic fever.

Sources of Material.

13 strains of hemolytic streptococci were isolated from patients with rheumatic fever, in most instances by means of throat swabs; and compared with them were 14 strains from other sources; these included the 4 type strains of Dochez, Avery, and Lancefield (6), (S3, S23, S60, and S84), Gordon's Type I *Streptococcus pyogenes* strain, a scarlet fever strain from Dochez (NY5), and an erysipelas strain from Dr. Harold L. Amoss (B3); the others were mostly from infections of the respiratory tract. These 27 strains were studied from the standpoint of sugar fermentation, agglutination, and agglutinin absorption. For the serological studies sera were prepared against 4 "rheumatic fever" and 5 other strains.

Table I shows the source of the strains and some of their cultural characteristics. Their ability to ferment carbohydrates was tested by growth in Hiss serum water with Andrade's indicator; the tubes were incubated for at least 4 days before being read; growth was verified by subcultures on blood agar plates. Special mention must be made of 3 of the "rheumatic fever" strains against which sera were prepared. T10 is of particular interest: it was recovered from an old rheumatic fever patient, A. M., a girl of 14, who was admitted

on account of a slight bronchitis. While under observation she developed acute angina in the course of an epidemic of sore throat which was present in the ward; from her throat nearly a pure culture of hemolytic streptococci (T10) was obtained. 4 weeks later she developed mild acute polyarthritis. 6 weeks after the onset of angina, large numbers of hemolytic streptococci (T30) were still present in her throat and, unlike most of the streptococci which Eagles studied from patients with scarlet fever, these seemed to be serologically identical with those recovered earlier. From another patient, A. B., a girl aged 16, dying of acute rheumatic carditis on the 16th day of her disease, streptococci (T20 and T25), apparently serologically identical, were recovered from the pericardium and tonsil respectively. T27b was a remarkable organism recovered from a subcutaneous fibroid nodule excised at biopsy from a boy aged 7. As shown in Table I, its sugar fermentations were unusual; it hemolysed blood on plates, but would never do so in liquid media; a final pH of 4.5 was attained in dextrose blood broth, as would be expected with a bovine strain. In addition to this strain only 4 of those studied failed to fall by their sugar fermentations into the classical *Streptococcus pyogenes* group.

Technique.

Sera were prepared by immunizing rabbits intravenously. The animals were given injections on 4 successive days, then 4 days rest, and so on; 3 courses were usually sufficient. For the first 6 injections the rabbits received heat-killed cultures which were increased gradually from 5 to 15 cc.; subsequently live cultures, starting with 0.5 cc. and increasing to 5 cc., were injected. 5 days after the last inoculation a test bleeding was made; if this was satisfactory the animal was exsanguinated and the serum stored on ice without preservatives. The sera all agglutinated the homologous organism to a dilution of 1:1280, and frequently to 1:2560.

The agglutination technique was similar to that described by Dochez, Avery, and Lancefield (6): buffered broth at a pH of 7.6 was used for the cultures and for making all dilutions; the sedimented organisms were washed once and resuspended in similar broth. Thus suspensions sufficiently stable for agglutination tests were obtained with all but 5 of the 27 strains. Unless the suspension was quite stable in the control tubes with broth and normal rabbit serum the experiment was disregarded. Readings were made after 1½ hours (in earlier experiments 2 hours) in the incubator at 56°C.

TABLE I.

Source of Streptococci and Action on Carbohydrates.

Strain	Patient	Disease	Organ	Abundance	Length of time between onset of sore throat and time of culture.	Lactose.	Saccharose.	Raffinose.	Inulin.	Salicin.	Mannitol.	Litmus milk.
					days							
T10	A.M.	Rheumatic fever.	Throat.	Nearly pure.	1	+	+	0	0	+	+	AC
T20	A.B.	"	Pericardium (autopsy).	Scanty. Mixed with green streptococci.		+	+	0	0	+	0	AC
T25	A.B.	"	Tonsil (autopsy).	Predominant organism.	32	+	+	0	0	+	0	AC
T27b	B.V.	"	Subcutaneous nodule.	Scanty. Green streptococci also recovered.		(±)	+	+	+	+	0	0
T30	A.M.	"	Throat.	50 per cent of colonies hemolytic.	42	+	+	0	0	+	+	AC
T40	A.McC.	"	"	About 8 hemolytic colonies on plate.	60	+	+	0	0	+	0	AC
T43	M.K.	"	Heart's blood (autopsy).	Pure culture.		+	+	0	0	+	0	AC
T61	S.R.	"	Throat.	Few colonies.	48	+	+	0	0	+	0	AC
T77	J.S.	"	"	Only 3 hemolytic colonies on a plate.	25	+	+	0	0	+	0	AC
T81	M.S.	"	"	Fair number of colonies.	16	+	+	0	0	+	0	AC
T88	A.T.	"	"		?	0	+	0	0	+	0	0
T102	M.S.	"	Tonsil.	Nearly pure cultures.	63	+	+	0	0	+	0	AC
T105	E.R.	"	Throat.	Very many colonies.	292	+	+	0	0	+	0	AC

Strain	Patient	Disease	Source	Remarks	No.							
S3	Texas patient 1918	Bronchopneumonia.	Lung (autopsy).			+	+	0	0	+	0	AC
S23	" "	Lobar pneumonia.	Throat.		?	+	+	0	0	+	0	AC
S60	" "	Measles.	"			+	+	0	0	+	+	A
S84	" "	Bronchopneumonia.	Pleural fluid.		?	+	+	0	0	+	0	A
Gordon Type I	London patient.	Abortion.	?			+	+	0	0	+	0	A
T58	P.A.	Lobar pneumonia.	Lung (autopsy).		1	+	+	0	0	+	0	AC
T70	G.M.	Sore throat.	Throat.	Nearly pure culture.		+	+	0	0	+	0	AC
T79	A.R.	Pneumonia.	Lung (autopsy).			+	+	0	0	+	0	AC
T92	C.N.	Sore throat.	Throat.	Many colonies.	7	+	+	0	0	+	0	AC
T95	E.E.	Septicemia.	Blood.	Pure culture.		+	+	0	0	+	0	AC
T99	F.Q.	Pneumonia.	"	"	3	+	+	0	0	+	0	AC
T107	R.T.	Sore throat.	Throat.	Many colonies.	?	+	+	0	0	+	0	AC
NY5	?	Scarlet fever.	"			+	+	0	0	+	0	A
Erysipelas B3	H.A	Erysipelas.	Blood.			+	+	0	0	+	0	AC

+ indicates fermentation with acid formation.

In the litmus milk column, A indicates acid formation and C clotting.

Agglutinin absorption was used as a check on the agglutination in all cases; by its aid we were able to study even the 5 strains in which the growth was persistently granular. Serum with a titre of at least 1:1280 was diluted 40 times with buffered broth; 0.75 cc. portions (in earlier experiment 1 cc.) of this diluted serum were each absorbed once with the centrifuged deposit from 50 cc. of heavy 18 hour growths of the various strains; these proportions were found sufficient to absorb the agglutinins from a serum by its homologous strain. After absorbing for 2 hours in a water bath at 37°C. the mixtures of serum and streptococci were centrifuged; the serum was pipetted off and set up against its homologous organism in dilutions of 1:80, 1:320, and 1:1280. Control tubes were always set up containing respectively a streptococcal suspension with broth, with normal rabbit serum, with unabsorbed immune serum, and with serum absorbed by the homologous organism. As it was found that live or heat-killed organisms were equally utilizable, living cultures were employed throughout. A typical protocol is shown in Table II.

TABLE II.

Protocol of an Agglutinin Absorption Experiment with S23 Serum.

All sera, both absorbed and unabsorbed, tested against Strain S23.

Serum dilution.	Absorbed by Strain S23.	Absorbed by Strain S60.	Absorbed by Strain S84.	Absorbed by Strain T20.	Absorbed by Strain T58.	Absorbed by Strain T61.	Absorbed by Strain T70.	Absorbed by Strain T77.	Absorbed by Strain T81.	Absorbed by Strain T88.	Absorbed by Strain T92.	Absorbed by Strain T95.	Absorbed by Strain T99.	S23 serum unabsorbed.	Normal serum.	Broth control.
1:80	0	4	4	4	4	4	4	4	4	4	4	4	4	4	0	0
1:320	0	4	4	4	4	4	4	4	4	4	4	4	4	4	0	
1:1280	0	3	3	3	3	3	4	4	3	4	4	3	2	4	0	

4 indicates complete agglutination and sedimentation; 3, complete agglutination with a little turbidity remaining above it; 2, lesser degrees of agglutination; 0, no agglutination.

RESULTS.

In Table III are shown the results in employing 9 sera to study 27 strains by means of agglutination and agglutinin absorption tests. As a rule, at least 2 rabbits were immunized against each strain; since there was never any significant difference in the behaviour of the 2 sera thus prepared against a single organism they are not represented separately in the table. The results obtained with the 2 tests are shown in parallel columns for purposes of comparison. 2 strains (T10 and T30) isolated from the same patient were serologi-

cally identical: T10 was, therefore, studied in greater detail than T30; the same remarks apply to T25, which was apparently the same as T20.

Several facts are at once apparent on studying the results of the agglutination and agglutinin absorption reactions. All strains were agglutinated by and absorbed agglutinins from their homologous sera; but in several instances there was a failure to obtain both reactions even though one of the two was positive. If it is necessary to have both agglutination and agglutinin absorption positive in order to identify any 2 strains as the same it is obvious that no 2 strains from our patients were identical, with the doubtful exceptions of T20 and T77. This seems to answer our original question; but incidentally a number of other points arose which are of general interest in the study of streptococci.

It is not uncommon to find streptococci which agglutinate with the sera prepared against other strains but which are distinct from those strains by the agglutinin absorption test. Thus Strains T92 and T107 were agglutinated by a number of antistreptococcus sera, but failed to absorb the agglutinins from these sera. On the other hand, the demonstration of agglutinin absorption in the absence of agglutination is unexpected. It was found that a scarlet fever strain (NY5) and at least 4 other strains absorbed the agglutinins from T20 serum, without being agglutinated by this serum, nor was Strain T20 agglutinated by serum prepared against either Strain NY5 or Strain S3. Durand and Sédallian (8) met with the same phenomenon on several occasions in the course of their studies on hemolytic streptococci; like us they also encountered a number of strains which were agglutinated by a given serum and yet failed to absorb the agglutinins from it.

Because of the close relationship found to exist between Strains S23, T10, and T40, careful study was given to them. Strain T10 fermented mannitol; the other 2 did not. Sera prepared against each of these strains agglutinated all 3 members of the group to a titre of 1:1280; hence, by agglutination they appeared to be identical. Each serum, on the other hand, had its agglutinins removed by absorption only with its homologous strain; absorption with the other 2 organisms did not lower the titre appreciably for that homologous

TABLE III.

Summary of Results of Agglutination and Agglutinin Absorption Tests.

Strains from rheumatic fever patients.	T10 Agg.	T10 Abs.	T20 Agg.	T20 Abs.	T27b Agg.	T27b Abs.	T40 Agg.	T40 Abs.	S3 Agg.	S3 Abs.	S23 Agg.	S23 Abs.	S60 Agg.	S60 Abs.	S84 Agg.	S84 Abs.	Gordon Type I Agg.	Gordon Type I Abs.
T10	+	AB	0	No	0	No	+	No	0	No	+	No	0	No	0	No	0	No
T20	0	No	+	AB	0	No	0	No	0	No	0	No	0	No	0	No	‡	No
T25	0	—	+	AB	0	—	—	—	0	—	0	—	0	—	0	—	0	No
T27b	0	No	0	No	+	AB	0	ab	0	No	0	No	0	No	0	No	—	—
T30	+	AB	‡	AB	0	No	—	—	0	—	+	No	0	—	0	No	—	—
T40	+	No	0	No	0	No	+	AB	0	No	+	No	‡	No	0	No	0	No
T43	0	ab	0	No	0	No	Gr.	No	0	No	0	No	0	No	0	No	Gr.	No
T61	0	No	‡	AB	0	No	Gr.	No	0	No	0	No	0	No	0	No	0	No
T77	0	No	Gr.	No	0	ab	0	No	0	No	0	No	0	No	0	No	0	No
T81	Gr.	No	+	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No
T88	0	No	Gr.	No	0	No	0	ab	0	No	‡	No	0	No	0	No	0	No
T102	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No
T105	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No
Strains from other sources.																		
S3	0	No	0	AB	0	No	0	No	+	AB	0	No	0	No	0	No	0	No
S23	+	No	0	No	0	No	+	No	0	No	+	AB	0	No	0	No	0	No
S60	0	No	0	No	0	No	0	No	+	No	‡	No	‡	AB	0	No	0	No
S84	0	No	0	No	0	No	0	No	0	No	‡	No	‡	No	+	AB	+	AB
Gordon Type I	0	No	0	No	0	ab	0	No	0	No	0	No	0	No	0	ab	0	ab
T58	0	No	0	No	0	No	0	No	0	No	0	No	0	No	0	No	0	No
T70	Gr.	No	Gr.	AB	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No

	Agg.	Abs.	Agg.	Abs.	Agg.	Abs.	Agg.	Abs.	Agg.	Abs.	Agg.	Abs.	Agg.	Abs.	Agg.	Abs.
T79	+	No	0	AB	0	No	0	No	0	No	0	No	0	No	0	No
T92	+	No	±	No	±	No	±	No	±	No	+	No	+	No	0	No
T95	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No
T99	0	No	±	No	0	No	0	No	0	No	0	No	0	No	±	No
T107	+	No	±	AB	0	No	+	No	+	No	+	No	+	No	0	No
NY5	0	No	0	No	0	No	0	No	0	No	0	No	0	No	0	No
Erysipelas B3	0	No	0	No	0	No	0	No	0	No	0	No	0	No	0	No

In the columns dealing with agglutination (Agg.) + indicates good agglutination up to dilutions of 1:1280 or higher; ±, agglutination in dilutions from 1:100 to 1:640; 0, no agglutination or a slight flocculation not over 1:80. Gr. indicates that the strain was too granular to work with.

In the columns dealing with agglutinin absorption (Abs.) AB means that nearly all the agglutinins were removed; a serum originally active at 1:1280 was inactive or almost so at 1:80. ab indicates that there was no agglutination at 1:1280 but strong agglutination at 1:320; the few results in this group probably belong with the group marked 0. No means no significant absorption; agglutination still occurred at 1:1280. − means that no test was carried out.

TABLE IV.

Table Showing Behaviour of S23, T10, and T40 Sera When Unabsorbed and When Absorbed by Their Homologous Strain.

	Serum dilution.	S23 serum unabsorbed.	S23 serum absorbed by S23.	T10 serum unabsorbed.	T10 serum absorbed by T10.	T40 serum unabsorbed.	T40 serum absorbed by T40.	Normal rabbit serum.	Broth control.
Sera tested	1:80	3	0	4	0	4	0	0	0
against Strain	1:320	4	0	4	0	4	0	0	—
S23.	1:1280	4	0	3	0	4	0	0	—
Sera tested	1:80	4	0	4	0	4	0	0	0
against Strain	1:320	4	0	4	0	4	0	0	—
T40.	1:1280	3	0	3	0	4	0	0	—
Sera tested	1:80	4	0	4	0	4	0	0	0
against Strain	1:320	4	0	3	0	3	0	0	—
T10.	1:1280	3	0	3	0	3	0	0	—

4 indicates complete agglutination and sedimentation; 3 complete agglutination with a little turbidity remaining above it; 0, no agglutination; —, no test made.

TABLE V.

Table Showing that Sera Absorbed by a Heterologous Strain Can Still Agglutinate the Homologous Organism but that This Treatment Removes All Agglutinins for the Absorbing Strain.

	Strains against which sera were tested.											
	Streptococcus S23.				Streptococcus T10.				Streptococcus T40.			
Serum dilution.	S23 serum absorbed by T10.	S23 serum absorbed by T40.	T10 serum absorbed by S23.	T40 serum absorbed by S23.	T10 serum absorbed by S23.	T10 serum absorbed by T40.	T40 serum absorbed by T10.	S23 serum absorbed by T10.	T40 serum absorbed by S23.	T40 serum absorbed by T10.	S23 serum absorbed by T40.	T10 serum absorbed by T40.
1:80	4	3	0	0	4	3	0	0	4	4	0	0
1:320	4	4	0	0	4	3	0	0	4	4	0	0
1:1280	4	3	0	0	4	4	0	0	4	4	0	0

For controls on this experiment see Table IV.

4 indicates complete agglutination and sedimentation; 3, complete agglutination with a little turbidity remaining above it; 0, no agglutination.

strain. By agglutinin absorption, then, each member of the group was distinct. This relationship is, of course, closely comparable with that existing between members of other more carefully studied groups of organisms such as the Salmonellas (*Bacillus paratyphosus* B, etc.). It seems necessary, therefore, to postulate the existence of specific and group antigens in these streptococci, as has been done by F. W. Andrewes in the Salmonellas (9). In order to determine whether 1 group antigen was common to these 3 streptococci the following series of experiments were performed: Each of the 3 diluted sera was divided into 3 lots; 1 portion from each serum was absorbed with Strain S23, 1 with T10, and 1 with T40; each of these absorbed

TABLE VI.

Table Showing Results of Absorbing a Serum with a Heterologous Strain and Then Testing the Serum against the Other Heterologous Strain.

Serum dilution.	Strains against which sera were tested.					
	Streptococcus S23.		Streptococcus T10.		Streptococcus T40.	
	T10 serum absorbed by T40.	T40 serum absorbed by T10.	S23 serum absorbed by T40.	T40 serum absorbed by S23.	S23 serum absorbed by T10.	T10 serum absorbed by S23.
1:80	4	4	4	0	4	0
1:320	1	1	4	0	4	0
1:1280	0	0	0	0	1	0

4 indicates complete agglutination and sedimentation; 1, lesser degrees of agglutination; 0, no agglutination.

sera was then tested for its capacity to agglutinate each of the 3 strains. The results are shown in Tables IV to VI.

Table IV shows that absorption of each serum by its homologous strain removed all the agglutinins from it.

Table V shows that absorption of a serum by a heterologous strain removed all agglutinins for the strain used for absorbing but failed to remove the specific homologous agglutinins; *i.e.*, those for the strain used in preparing the serum. The unabsorbed serum and normal serum controls are recorded in Table IV, hence are not repeated in Tables V and VI.

So far the results are not unexpected; but those in Table VI are more interesting. If the cross-agglutinations among these 3 strains

were due to 1 group antigen common to all 3, absorption of any serum by a heterologous strain should remove the group agglutinins for the other heterologous strain.

But it is evident that when Strain S23 was used for absorbing, all "group" agglutinins were removed, as was expected; on the other hand, when Strains T10 and T40 were used for absorbing, the titre of the serum for the heterologous strains was only slightly reduced; the experiment was twice repeated with similar results. When the absorbing doses used were 10, and even 26 times the ordinary dose employed, still T10 serum absorbed by T40 and T40 serum absorbed by T10 were able to agglutinate S23 almost as well as originally. Streptococcus S23 varied from day to day in the degree to which it was agglutinated by T10 and T40 sera; hence it cannot be concluded that any absorption had occurred in the experiment recorded in the first 2 columns of Table VI, for on the date of this experiment it did not agglutinate any better with unabsorbed sera.

In trying to determine whether each strain contained an antigenic element not shared by either of the other 2, the same sample of each serum was absorbed successively by the 2 heterologous strains. In no case did this procedure affect the titre of the serum against its homologous strain. A strain, S65, isolated by Dochez, Avery, and Lancefield (6) in 1918 and found by them to be a homologue of S23 behaved towards Strains T10 and T40 exactly as did Strain S23, both by agglutination and agglutinin absorption tests.

To interpret the foregoing results it seems necessary to assume that each streptococcus contains a specific antigen and at least one other factor which is common to all. The results shown in Table VI, moreover, make it appear that the complete explanation is somewhat complex. It is possible that we have to deal with more than one group antigen, as seems to be the case with the Salmonellas (10); but this is by no means proven. It is also possible that the 3 organisms under study differ in the relative amounts of group and specific antigens which they contain, or that the situation is complicated by other factors as yet not understood. In any case, it would be premature, after a study of only 3 strains to try to portray graphically their antigenic mosaic, though it is tempting to endeavour to do so. The results obtained, however, indicate that hemolytic streptococci may be antigenically related to each other in a rather complex manner.

It has been shown that the members of the Salmonella groups are diphasic (F. W. Andrewes (9, 10)) because some individuals of each strain possess mainly group antigen while others have almost wholly the specific antigen.   It seemed desirable, therefore, to pick individual colonies of the 3 streptococcal strains under study and to see if cultures from each colony behaved alike towards the various sera. Broth cultures from 18 colonies of Strain S23, 15 of T40, and 33 of T10 were tested by agglutination against all 3 sera; all behaved alike within narrow limits.   The agglutinin absorption test was not applied to the cultures from these colonies; one cannot, therefore, say definitely that they were antigenically identical, but can only presume that all the colonies picked contained at least one common antigen.

Towards the end of this work we encountered another illustration of the difficulties which beset the worker who studies streptococci. It was necessary to prepare a fresh supply of serum against Strain T10: this was found to have as high a titre against T10 as our original serum; it had, however, only a small amount oi agglutinins for Strains S23 and T40 although, as will be shown later, it apparently had good protective power against Strain S23 when tested in mice (see Table VIII).   The stock blood broth culture which was kept in the ice box and used in preparing this serum was formerly agglutinated by T10, S23, and T40 antisera in dilutions of 1:1280; on retesting, we found that it still agglutinated to 1:1280 with its homologous serum, to 1:1280 with T40 serum, and to 1:320 with S23 serum.   On the other hand some of the original culture which had been preserved by drying in the frozen state was still agglutinated with T40 and S23 sera in dilutions of 1:1280, as of old.   It thus appears that variations may occur in the power of a culture to show cross-agglutination and to stimulate the production of group agglutinins even though we were unable by picking colonies to demonstrate the existence of sharply defined phases.

The agglutination and agglutinin absorption tests each considered separately would lead to very different conclusions as to the relationships existing among S23, T10, and T40.   It seemed of interest, therefore, to compare the relationships indicated by these 2 methods with those demonstrated by protection experiments in mice.   In their study of hemolytic streptococci, Dochez, Avery, and Lancefield

(6), whenever they could raise the virulence of a strain high enough to study mouse protection, were able to confirm by means of protection their classification based on agglutination. They did not

TABLE VII.

Protective Power of S23, T10, and T40 Sera against S23 (First Experiment).

	Doses of streptococci.					
	0.1 cc.	0.01 cc.	0.001 cc.	0.0001 cc.	0.00001 cc.	0.000001 cc.
Unprotected mice.....	†20 hrs.	†40 hrs.	†65 hrs.	†72 hrs.	†65 hrs.	†14 days.
Mice protected by S23 serum..............	S.	S.	S.	S.	S.	S.
Mice protected by T10 serum..............	"	†26 hrs.	†40 hrs.	"	"	"
Mice protected by T40 serum..............	†20 hrs.	†30 "	†30 "	"	"	"

S = survived.
† = died.

TABLE VIII.

Protective Power of S23, T10, T40, and Gordon's Type I Sera against Strain S23.

	Doses of streptococci.						
	0.1 cc.	0.01 cc.	0.001 cc.	0.0001 cc.	0.00001 cc.	0.000001 cc.	0.0000001 cc.
Unprotected mice...	†10 hrs.	†20 hrs.	†22 hrs.	†46 hrs.	†20 hrs.	†22 hrs.	†46 hrs.
Mice protected by S23 serum.......	†20 "	†20 "	S.	S.	S.	S.	S.
Mice protected by T10 serum........	†22 "	†20 "	"	"	"	"	"
Mice protected by T40 serum........	†20 "	†20 "	†20 hrs.	†22 hrs.	"	"	"
Mice protected by Gordon's Type I serum...........	†20 "	†20 "	†46 "	†20 "	†46 hrs.	†24 hrs.	†46 hrs.

S = survived.
† = died.

study agglutinin absorption; but some of their old strains were fortunately available for our use. Strains S10 and S100 were found by them to be homologues of S60, Strains S15, S50, and S110 were homologues of S84, and Strain S65 was the same as S23. We ap-

plied the agglutinin absorption test to these strains and in all cases the results confirmed the classification made by these workers. In other words, with the strains they studied the results based on agglutination, agglutinin absorption, and mouse protection are identical. Only 1 of our strains, S23, could be rendered sufficiently virulent by repeated passage through rats and mice to be of value in mouse protection tests; it finally killed mice in doses of 0.000001 to 0.0000001 cc. We employed the same technique for our protection tests as that used by the authors mentioned above: mice were given 0.5 cc. of immune serum intraperitoneally and 24 hours later were inoculated by the same route with the appropriate dose of culture suspended in 0.5 cc. of normal salt solution.

The protocols of 2 experiments are given above.

It will be seen that the homologous serum protected mice against 0.1 cc. of S23 culture in the first and 0.001 cc. in the second experiment; these results are perhaps comparable as the second culture used was more virulent. In the first experiment T10 and T40 antisera each showed some protective power, in that they saved the mice from 0.0001 cc. of culture. In the second experiment, however, T40 antiserum only protected against 0.00001 cc. of culture as might be expected because of the increased virulence of the strain; but in the case of T10 antiserum there was protection equal to that afforded by S23 antiserum. This was unexpected since we used, in this test, a fresh lot of T10 serum which agglutinated Strain S23 very feebly. Serum prepared against Gordon Streptococcus Type I, an organism with which Strain S23 showed no cross-agglutination, had no protective power against the latter strain. This is in agreement with Dochez, Avery, and Lancefield (6), who found no evidence of protection by completely heterologous sera. If we had attempted to classify our 3 strains solely by the mouse protection method there would have been some doubt whether T10 and more doubt whether T40 should be included in the same group with S23.

DISCUSSION.

Our inability to demonstrate a serological relationship among hemolytic streptococci from patients with rheumatic fever needs no further discussion. We realize, of course, that this failure would

be more convincing if we could have obtained more cultures at the time of the onset of the sore throat.

Without preparing many more sera it is not possible to state that all the streptococci studied were serologically distinct; we have not attempted a complete classification of the 27 strains. It is interesting however, to note that we met with no strain in New York City which corresponded with any of the 4 type strains which Dochez, Avery, and Lancefield encountered in Texas or with Gordon's Type I strain, which he believes to include the majority of *Streptococcus pyogenes* (3). Our failure to find strains similar to those isolated in Texas is not surprising in view of the fact that Valentine and Mishulow (11) in New York during 1921 were able to isolate from 52 streptococcus-infected patients only 1 strain identical with any of the 4 type strains of Dochez, Avery, and Lancefield; in their studies both agglutination and agglutinin absorption tests were applied. Krumwiede, Cooper, and Provost (12) have advanced arguments indicating that reciprocal agglutinin absorption is the ultimate criterion of serological likeness of two bacterial strains. Our work indicates that such agglutinin absorption must be used to control the results of agglutination. We have not admitted the identity of any 2 strains unless the results of these 2 tests agree; there seems at present no reason for applying to the classification of the hemolytic streptococci criteria less rigid than are employed with other groups of bacteria. The results obtained with Strains S23, T10, and T40 raise the whole question of the relative value of agglutination, agglutinin absorption, and protection for the classification of streptococci. In spite of much labour the classification of the hemolytic streptococci is in its infancy. It is a study complicated by the fact that, although complement fixation (Kinsella and Swift (13)) and precipitin reactions (Hitchcock (14)) might seem to indicate that the group is a homogeneous one, yet agglutination, agglutinin absorption, and mouse protection methods tend to show that it is heterogeneous. The explanation of the results obtained by the complement fixation and precipitin tests is probably to be found in the existence of identical or closely related nucleoprotein and soluble substance elements in different strains (Lancefield (15); Hitchcock (14)). It seems not unlikely that, as Durand and Sédalian (8) suggest, streptococci do not fall into sharply defined types

but contain an antigenic mosaic, the constituent parts of which are distributed in a more or less complicated manner amongst the members of the class.

SUMMARY.

1. Thirteen strains of hemolytic streptococci isolated from patients with acute rheumatic fever did not show any evidence of being related serologically.

2. Strains of hemolytic streptococci exist which, although identical by the agglutination test, do not show complete reciprocal agglutinin absorption.   Sera prepared from such related strains may show some protective power in mice against another member of the group.

3. Some strains of hemolytic streptococci may absorb agglutinins from a serum prepared against another strain, and yet may fail to be agglutinated by that serum.

4. None of 20 strains of hemolytic streptococci isolated in New York City in 1924, and 1925, corresponded serologically with Dochez, Avery, and Lancefield's 4 type strains or with Gordon's Type I strain.

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THE PRODUCTION OF PURPURA BY DERIVATIVES OF PNEUMOCOCCUS.

I. GENERAL CONSIDERATIONS OF THE REACTION.

BY LOUIS A. JULIANELLE, PH.D., AND HOBART A. REIMANN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

During the course of studies on oxidation and reduction by pneumococcus made by Avery and Neill (1), Neill made the observation that when pneumococcus extract was injected into white mice hemorrhagic purpura developed. At a later date, we made a similar observation. This paper is a report of the study of the nature of this phenomenon.

In 1907, Heyrovsky (2) reported that he was able to produce purpura in white mice by injecting 24 hour filtrates of virulent pneumococci grown in dextrose broth. No other reference to this reaction has come to our attention.

We have found that if pneumococcus extracts, prepared by one of the various methods to be described later, are injected into white mice, after 4 to 6 hours the skin over the feet, tail, ears, snout, and genitalia take on a dark bluish purple color. This peculiar appearance is confined to the areas where the hair is either absent or scanty, but removal of the hair does not render the skin in the shaved area reactive. The intensity of the discoloration varies in the different regions and only one or several or all of the sites mentioned may be involved. Unless the amount of extract injected is very large the animals show no signs of intoxication and all recover. The lesion is at its maximum intensity usually in 24 to 48 hours following the injection and then slowly disappears, vanishing entirely in 5 to 7 days.

The development of the reaction may best be observed in the ear by transmitted light. It is then seen that the lesion begins as a small hemorrhage about the blood vessels and the extravasation extends into the surrounding tissues.

The microscopical changes[1] are best studied in sections made of the skin and viscera of animals autopsied immediately after they have died or been killed by chloroform vapor.   It is then found that the changes are not confined to the skin but are also present in the lungs and voluntary muscles.   The essential lesion consists of extravasations of blood into the loose tissues surrounding the blood vessels. In the skin the hemorrhages are usually found in the subcutaneous tissues, but may also be present in the superficial layers of the corium. The hemorrhages may be minute or may involve quite extensive areas. Frequently small collections of polymorphonuclear leucocytes are present in the hemorrhagic areas.

The degree of purpura is influenced both by the amount of the material introduced and the individual susceptibility of the experimental animal.   Using a fixed quantity of extract for a series of mice there is considerable variation in the severity of the reaction in the individual animals.   0.2 cc. of extract usually produces purpura in half of the mice of a series.   0.4 cc. gives 90 per cent or more positive results with rather severe reactions.   More than this amount proves too toxic and death usually occurs, although some mice have been able to withstand 0.6 cc. and, rarely, 0.8 cc. of the extract.

The reaction is produced equally well whether the extract is introduced subcutaneously, intraperitoneally, or intravenously.   The intravenous method usually results in a more rapid reaction.   Attempts to effect purpura by feeding have not, however, been successful.   After being subjected to chloroform, mice were fed by stomach tube with amounts much greater than necessary to cause purpura if injected, but in these cases no purpura was evidenced.   This is not surprising since trypsin destroys the activity of the purpura-producing principle.

Purpura was produced in guinea pigs and rabbits also.   In the guinea pig the ears and the pads of the feet were affected; while the ears and scrotum of male rabbits,—which alone were used,—were chiefly involved.

Derivatives of Pneumococcus Causing Purpura.

In our study the development of purpura was first observed in white mice as a response to injections of pneumococcus extract pre-

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[1] The pathology of the effect of pneumococcus extract in experimental animals is under study by Dr. Branch and a report upon it will be made subsequently.

pared according to the method of Avery and Neill (3). Purpura was also produced in white mice by Heyrovsky's method. Cultures of pneumococcus were grown in dextrose broth and a 1 day's growth was filtered and injected intraperitoneally. The reaction, however, was much less constant and less severe than that obtained by the introduction of extract. Moreover, when purpura did appear, as much as 1.5 to 2.0 cc. were required to produce the reaction. Filtrates of young cultures (8 to 16 hours) grown in plain beef infusion broth, however, have never yielded purpuric lesions. Filtrates from very old cultures grown in plain infusion broth for 3 weeks only rarely have given purpuric reactions and when they did the activity was slight.

Whole cultures, unfiltered, rarely cause the purpuric reaction. Young cultures usually cause an infection followed by rapid death of the mice. In hundreds of mice injected with young cultures of pneumococcus for one purpose or another never but once has purpura followed the injection. On this occasion the mouse had been injected with a virulent culture together with sufficient immune serum to afford passive protection. On the 5th day following the injection purpura was noted. It increased in intensity until the 7th day and then began to blanch. The infection itself, however, does not prevent the appearance of purpura, for purpura results when an active extract is injected into an animal together with a minimum infecting dose of culture.

Old cultures occasionally produce purpura. Two strains of Type III have given the most consistent results. After the original 24 hour culture in blood broth had been stored for a week or more in the ice chest, these strains were able to produce purpuric changes. Since the mice were not severely infected, it is probable that during storage the cultures had undergone sufficient autolysis to be partially transformed into extract-like substances.

Bile-dissolved cultures have never induced purpura, even when solutions prepared from young cultures of pneumococci, concentrated to the same extent as is done in preparing extracts, and dissolved in a minimum quantity of bile, are employed. That bile in itself does not inhibit the purpurogenic activity of an extract was shown by adding bile to an active extract in a concentration of 10 per cent. This mixture injected into mice produced purpura.

Pneumococci which had been killed by exposure to a temperature of 56°C. for 30 minutes,—a treatment sufficient to kill the pneumococci but not to destroy the activity of the purpura-producing material,—did not produce purpura in white mice. The soluble specific substance and nucleoprotein of pneumococcus (Heidelberger and Avery (4)) failed to produce purpura regardless of the quantity injected.

The observations so far recorded suggest that the substance, which on injection gives rise to purpura, is contained in the bodies of the pneumococci and is set free by the process of extraction employed. It is also found occasionally in the culture filtrate. It has been of importance to determine whether this substance exists preformed in the bacterial cell or whether it is a product of a digestive process occurring during the process of extraction.

Concentrated suspensions of pneumococci were prepared by centrifuging 1500 cc. of a 9 hour broth culture and suspending the sediment in 15 cc. of broth. When trypsin was allowed to act on a portion of this concentrated suspension the resulting fluid was not active. This was the case whether the bacteria in the emulsion were alive or had been killed by heat before exposure to the action of trypsin.

If, instead of trypsin, the native enzymes of the pneumococcus were allowed to act on the concentrated emulsion, the results were quite different, since the product in this case was highly active. In this experiment to 15 cc. of the concentrated pneumococcus emulsion, 10 cc. of an homologous extract containing the pneumococcus enzymes (Avery and Cullen (5)) were added and the mixture was incubated for 40 hours at 37°C. The mixture was then centrifuged and the supernatant fluid filtered. This filtrate was now found to be as active as the pneumococcus extract prepared by the Avery-Neill method.

The Nature of the Purpura-Producing Principle.

Aliquot portions of pneumococcus extract were heated at 60°C., 70°C., 80°C., and 100°C. for 10 minutes each. At the higher temperature exposures, coagulation occurred. The various specimens were injected individually into the peritoneum of white mice and it was observed that heating even at 100°C. for 10 minutes did not destroy the activity of the purpura-producing material. The results are appended in Table I.

Avery and Neill were able to destroy both the hemolytic and oxidation-reduction activity of extracts by exposure to air. It was of

interest to determine, therefore, the effect of oxidation on the purpura-producing principle of the extracts. Pneumococcus extract was oxidized until it was no longer hemolytic. It was still capable of producing purpura when injected into white mice. It can be said, then, that the purpura-producing principle is not associated with the hemolytic activity of the extracts, since the latter alone is destroyed by heat and oxidation.

Extracts of all groups of pneumococci contain the purpura-producing material. Experiments performed to correlate the purpura-producing activity with virulence showed no relationship between these two properties. An avirulent Group IV strain gave as good a purpura-producing extract as any of the most highly virulent strains in this laboratory.

TABLE I.

The Effect of Heat on the Purpura-Producing Material.

Each mouse was injected intraperitoneally wth 0.4 cc. of the respective extract.

Type of extract.	No. of mice in test.	No. of mice developing purpura.
Unheated extract....................................	4	3
Extract heated 10 min. at 60°C.,......................	2	2
" " 10 " " 70° "	2	2
" " 10 " " 80° "	2	1
" " 10 " " 100° "	6	5

Because it has been shown (6, 7) that an avirulent culture of pneumococcus may be a strain composed of both virulent and avirulent organisms, the experiment was extended to include a study of the extracts prepared from the R or purely avirulent strains. It was shown that the extracts possessed purpura-producing material.

Isolation of the Purpura-Producing Material.

For the isolation of the purpura-producing material from the extracts, saline extracts of pneumococcus were prepared according to the technique of Avery and Neill. These extracts were equivalent to the broth extracts in producing purpura. Normal acetic acid was added slowly to the saline extract until no further precipitation occurred. The material was centrifuged and all of the supernatant was withdrawn. The sediment was washed with normal salt solution, then dissolved in weak alkali and neutralized, and the volume was adjusted to the original

quantity.   The supernatant no longer gave a precipitate with acetic acid.   It was neutralized with normal NaOH.

The supernatant was next half saturated with $(NH_4)_2SO_4$ and a precipitate was obtained.   This was centrifuged and the supernatant was collected and made up to full saturation with $(NH_4)_2SO_4$ and another precipitation was obtained.

The acetic acid precipitate, which contained essentially the nucleo-proteins of pneumococcus (4) produced no purpuric lesions when injected into white mice.   This was in accordance with the earlier observation on the nucleoprotein fraction.   The supernatant from the acetic acid precipitation, on the other hand, was still able to pro-duce purpura with apparently no loss in this property.   The precipi-tate obtained by half saturation with $(NH_4)_2SO_4$ did not contain the purpura material while the supernatant was still purpura-producing. The precipitate obtained by full saturation with $(NH_4)_2SO_4$ was shown to contain the purpura-producing principle.   This precipitate was washed free of salts by dialysis.

It was found that the purified purpura-producing principle was co-agulated on heating and was precipitated by trichloracetic acid, sulfo-salicylic acid, and alcohol.   It gave a positive biuret reaction, but did not give a xanthoproteic reaction and the Millon reaction was a doubtful one.   The activity to cause purpura was destroyed by trypsin digestion.

The purified material acted like the extracts themselves and mice showed the same degree of variation in susceptibility to it that they did to the extracts.   As small an amount as 0.04 mg.,[2]—a dosage of about 1/500,000 of the body weight,—was sufficient to produce purpura in some of the mice.   It proved to be highly toxic for rabbits, and 0.16 to 3.2 mg. was fatal to 3 rabbits.   The purpura-producing substance was not precipitated either by anti-extract or antipneumo-coccus sera.

Purpuric Responses to Extracts from Other Bacteria.

Experiments were conducted to determine whether the purpura-producing material was peculiar to pneumococcus alone.   Extracts

[2] These quantities were calculated as protein on the basis of the organic nitrogen in the solution obtained by full saturation of the extract with $MgSO_4$, after precipitation with acetic acid.

were made of two strains of *Staphylococcus aureus*, three strains of *Streptococcus viridans*, one strain of hemolytic streptococcus, and one strain of *Bacillus coli*.

For these organisms[3] the preparation of the extracts was modified, since rapid freezing and thawing did not give solutions of the bacterial cells. The cultures were centrifuged and to the sediment was added a small amount of sterile sand. The bacteria were then dried *in vacuo* at a temperature not exceeding 37°C. and then ground in a ball mill at room temperature for varying periods of 48 hours or more until a fine powder was obtained. At this stage N/100 alkali was added and extraction was allowed to take place in the ice chest, in the ball mill. On the following day the material was removed and centrifuged and the supernatant was used in the experiments. Care was taken to keep the concentrations equivalent to that of the pneumococcus extract.

Extracts were prepared from four strains of meningococcus by freezing and thawing.

The extracts from these organisms were not capable of producing purpura in white mice. Amounts as great as 2.5 cc. were used for injection.

DISCUSSION.

Evidence has already been presented to show that pneumococcus extracts possess a powerful hemolysin and an oxidation-reduction system which is operative on hemoglobin (Avery and Neill). The present paper offers data to show that the extracts possess in addition a toxic principle which is capable of producing experimental purpura in animals. This activity is derived from extracts of avirulent as well as of virulent pneumococci. Extracts of the other bacteria tested either do not possess this activity, or the methods which were applicable for pneumococcus were inadequate to demonstrate it.

The purpura-producing material is obtained from the extracts by full saturation with $(NH_4)_2SO_4$. Whether it is an albumin or some closely related substance as a higher proteose, for example, for which albumin may act merely as a vehicle remains to be determined.

It is significant that bile solutions of pneumococci did not yield the purpura-producing substance. That, on the other hand, bile does not prevent its activity has also been shown. And those constitu-

[3] We are indebted to R. C. Lancefield for this method.

ents of the pneumococcus which exist preformed in the cell are recoverable by dissolution of the cell with bile. The method has been used successfully by Cole (8) to obtain the hemolysin, and by Avery and Cullen (9) to obtain the enzymes of pneumococcus. The purpuric substance was found to be present in those materials alone which represented degradation products of pneumococci. It appears probable, therefore, that the purpura-producing property is due not to a constituent of the living pneumococcus cell, but to a degradation product of pneumococcus. That it is a separate entity from the pneumococcus hemotoxin has been shown selectively. The hemotoxin can be eliminated by oxidation and heat, while neither of these means destroys the activity of the purpura-producing material. Autolysates of pneumococcus are definitely purpura-producing, yet, as prepared by our method, they contain no demonstrable hemotoxin. The fact that autolysates were purpura-producing while autolyzed cultures were rarely, and at best, poorly purpura-producing, is explainable on the basis of concentration, since the autolysates were 50 times more concentrated than the cultures.

It is well recognized that purpura accompanying pneumococcus infections in man is of extreme rarity. There are, however, occasional instances recorded in the literature. Bazan (10) and Nobécourt and Mathieu (11) have reported upon them. In their cases the purpura always preceded death. Whether there is any relation between the purpura-producing principle and the pneumococcus infections cannot be said at the present time. It is quite possible that the purpura substance may function in infections without necessarily producing visible signs of purpura. Experiments are planned to determine whether the purpura principle plays any part in pneumococcus infections.

CONCLUSIONS.

1. Pneumococcus extracts have been shown to be capable of producing hemorrhagic purpura in white mice, rabbits, and guinea pigs.

2. The purpura-producing principle resists heating to 100°C. for 10 minutes; it resists oxidation; it is filter-passing; its activity is destroyed by digestion with trypsin; it is obtained from pneumococcus extracts by full saturation with $(NH_4)_2SO_4$, after the acetic acid-precipitable substances have been removed from the extracts.

3. The purpura-producing principle is common to all four types of pneumococcus and apparently bears no relation to virulence.

4. The purpura-producing principle is probably a degradation product of pneumococcus.

5. This principle is not associated with the hemotoxin of pneumococcus, since the hemolytic activity of an extract may be destroyed without effect on the ability to cause purpura.

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THE PRODUCTION OF PURPURA BY DERIVATIVES OF PNEUMOCOCCUS.

II. THE EFFECT OF PNEUMOCOCCUS EXTRACT ON THE BLOOD PLATELETS AND CORPUSCLES.

BY HOBART A. REIMANN, M.D., AND LOUIS A. JULIANELLE, PH.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

It has been shown (1) that pneumococcus extracts produce purpuric lesions in white mice, rabbits, and guinea pigs. A number of toxic substances (benzene, saponin, diphtheria toxin, etc.) also have the property of producing purpura in experimental animals. It appears from previous studies that a combination of factors is involved in the production of experimental purpura. One of them is the excessive diminution in the number of blood platelets, which may be due either to their actual destruction, or damage to their seat of origin; the other is injury to the capillary walls. It seemed of interest, therefore, to study the variations in number of the blood platelets in white mice which were injected with pneumococcus extract.

Method Employed in Counting Platelets.—It was not possible to count the platelets by the direct method on account of the small quantities of blood available in mice. The indirect method was therefore adopted, in spite of the errors inherent in it, and was found satisfactory in that it gave a conception of the approximate number of blood platelets present.

The mouse's tail was warmed over an electric bulb to insure a free flow of blood. The tail was then laid on a block of wood with the tip immersed in normal salt solution containing 2 per cent sodium citrate. A short piece of the tail was cut off with a razor blade, and as the blood began to flow, the tail was removed from the citrate solution, and touched to a clean glass slide. The drop was spread over the surface with the edge of another slide. Four slides were made for each count. Haste to prevent clumping of the platelets was not necessary because of the presence of the anticoagulant. More blood was taken for erythrocyte and leucocyte counts and the bleeding was stopped by searing. The slides for the platelet counts were stained with Wright's stain in the usual manner. After

the relative number of blood platelets and erythrocytes had been determined from the stained smears, and the actual number of erythrocytes had been found, the number of blood platelets was determined by proportion.

Care was exercised to prevent an unnecessary amount of bleeding. Control counts after similar repeated bleedings in normal mice showed no marked changes referable to the loss of blood.

EXPERIMENTAL.

The average number of erythrocytes in normal mice was found to be 8,000,000 and the platelets 1,800,000 per c.mm.

Effect on Platelets.—The mice were injected intraperitoneally with doses of 0.3 to 0.6 cc. of extracts obtained from Group IV, Type II, Type III, and the variant form of Type I pneumococcus. The method of preparation of these extracts has been described by Avery and Neill (12). All gave similar results. Counts were made at varying intervals, from half an hour to several days. Variation in the platelet count was observed in twenty mice.

A rapid fall in the number of platelets occurred soon after the injection of the extract. In one instance a third of the total number disappeared within 20 minutes. Usually the greatest diminution occurred after 24 hours. The lowest count obtained was about 8000 per c.mm. The number increased subsequently in the mice that survived the effects of the extract and reached normal in from 4 to 9 days. Thereafter there was an increase above normal lasting 2 to 12 days and by the 14th to the 20th day there was a return to normal which was maintained. In mice which failed to show purpura the platelets did not fall below 800,000 per c.mm. As a rule, purpura occurred only when their number was less than 500,000 per c.mm. Some specimen results are recorded in Tables I and II and illustrated by Graphs 1 and 2.

In general, the curves obtained by plotting the number of blood platelets in white mice, following the injection of pneumococcus extract, were similar to those obtained during the course of pneumonia, a number of other infectious diseases, and following the injection of certain toxic substances or antiplatelet serum in experimental animals. The initial reduction in the number of blood platelets was followed by a rise which exceeded the normal count, and later returned to normal.

In order to determine whether a cumulative effect on the platelets

could be obtained, one mouse was given five daily injections of small doses of the extract (0.05 cc. daily). During the course of these injec-

TABLE I.

Changes in Platelets and Erythrocytes Following Injection of Pneumococcus Extract.

Mouse III injected intraperitoneally with 0.4 cc. of Type II extract.

Time of examination.	No. of platelets.	No of erythrocytes.	Remarks.
Before injection..............	1,500,000	8,130,000	
20 min. after injection......	1,000,000		
1 hr. " "	700,000		
2 hrs. " "	510,000		Slight purpura in right ear.
4 " " "	500,000	5,240,000	Purpura in feet and tail also.
24 " " "	160,000	4,300,000	Purpura in feet, tail, and ears.
52 " " "	140,000	4,300,000	" " "
96 " " "	240,000	2,400,000	Purpura disappearing.
108 " " "	810,000	2,240,000	No purpura observed.
192 " " "	2,000,000	2,900,000	
9 days " "	2,800,000	4,100,000	
12 " " "	2,100,000	4,700,000	
18 " " "	1,600,000	7,300,000	

TABLE II.

Changes in Platelets and Erythrocytes Following Injections of Pneumococcus Extract.

Mouse VI injected with 0.4 cc. Group IV extract.

Time of examination.	No. of platelets.	No. of erythrocytes.	Remarks.
Before injection..............	2,000,000	8,200,000	
90 min. after injection.......	1,400,000		
4 hrs. " "	640,000		Purpura in right ear.
24 " " "	74,000	5,800,000	Severe purpura in both ears feet, and tail.
72 " " "	750,000	4,200,000	Purpura disappearing.
5 days " "	3,000,000	5,900,000	Slight trace of purpura left.
9 " " "	5,700,000	8,200,000	
14 " " "	3,700,000	6,500,000	
21 " " "	2,000,000	8,000,000	

tions there was a gradual diminution in the number of blood platelets to 750,000 per c.mm., after which the number began to return toward the normal, when the experiment was discontinued

Camera lucida studies did not reveal the variations in size of the platelets which have been observed during the course of pneumonia in man (2).
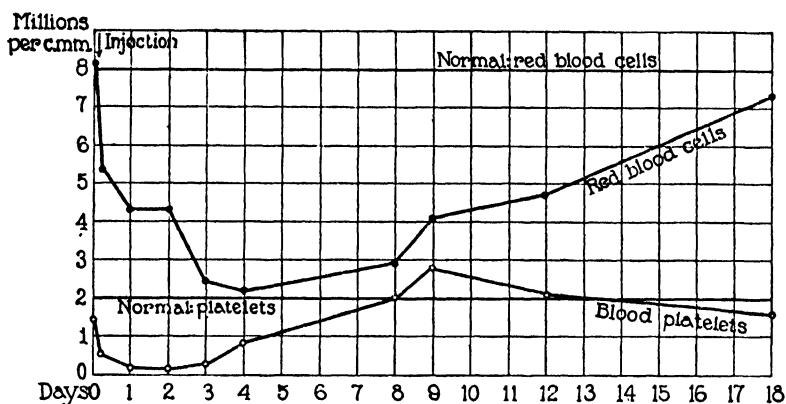
GRAPH 1. The effect of pneumococcus extract on the number of blood platelets and red cells. Graph illustrating Table I.

The mouse of 15 gm. was injected intraperitoneally with 0.4 cc. Type III extract.
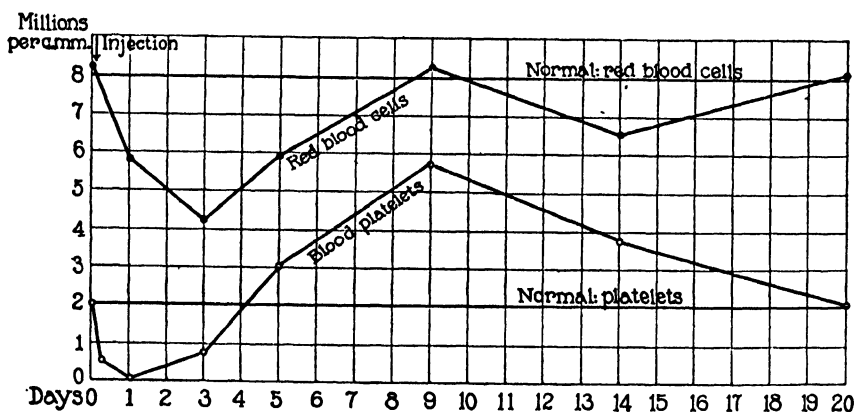
GRAPH 2. The effect of pneumococcus extract on the number of blood platelets and red cells. Graph illustrating Table II.

The mouse of 15 gm. was injected intraperitoneally with 0.4 cc. Group IV extract.

Effect on Erythrocytes.—Injections of pneumococcus extract caused a marked reduction in the number of erythrocytes as well as in the number of platelets as shown in Tables I and II. The rate of dim-

inution was somewhat slower for erythrocytes, but the diminution continued even after the blood platelets had begun to increase in number. The reduction of erythrocytes was most marked in from 3 to 7 days, and there was a return to normal in 10 to 20 days. The diminution of erythrocytes usually amounted to 50 per cent or more. That this anemia was not entirely referable to hemorrhage into the tissues is concluded from the fact that anemia may follow injections of pneumococcus extract in the absence of a visible purpura. Conversely, severe purpura may develop without the occurrence of a severe anemia as will be shown further on.

An occasional mouse showed a marked lipemia following severe anemia. This has also been observed by Bloor (3) and Horiuchi (4), in experimental animals in which anemia had been induced artificially.

Effect on Leucocytes.—These cells did not show marked fluctuation. Frequently a slight leucocytosis was observed immediately after the injection of the extract, followed by a slight leucopenia.

Observations on the Lytic Action of Pneumococcus Extract.

The rapid diminution in the number of platelets and erythrocytes in the injected mice suggested that the destruction might be due to direct lysis of the cells. Accordingly the lytic effect of the extract was tested *in vitro*.

Human and rabbit platelets were obtained by rapidly running 30 cc. of blood into 3 cc. of saline solution containing 5 per cent sodium citrate. The mixture was centrifuged slowly for 5 minutes to sediment the red and white blood cells. The supernatant fluid was separated and centrifuged at high speed to obtain the blood platelets. The blood platelets and erythrocytes were washed separately twice, and each sediment suspended in saline. In performing the lytic tests, 0.5 cc. quantities of extract, suspensions of platelets, and erythrocytes were used.

It was found that the extract was lytic for platelets and erythrocytes in dilutions as high as 1:100 when incubated with them for 1 hour at 37°C. However, the lytic action was completely prevented when both platelets and erythrocytes were suspended in serum instead of salt solution. Blood platelets which had been suspended in serum and stronger concentrations of the extract appeared blurred when stained and examined under the microscope. No lytic action against leucocytes was demonstrated *in vitro*.

Similar observations were made when bile, saponin, or sodium oleate were employed as lytic agents. The bile we used was lytic for platelets in dilutions of 1:600, the saponin in dilution of 1:200,000, and the sodium oleate in dilution of 1:100,000. In the same or somewhat greater concentrations, however, none of these agents was lytic for platelets when suspended in serum.

Observations were made *in vitro* on the effect of heat on the thrombolytic activity of pneumococcus extracts. The extract was heated at 55°C. for 10 minutes; and titrations of it were made before and after heating. The results are shown in Table III.

TABLE III.

The Effect of Heated and Unheated Pneumococcus Extracts on Platelets and Erythrocytes in Vitro.

Dilution of extract.	Unheated extract.				Extract heated 10 min. at 55°C.			
	Platelets suspended in		Red blood cells in		Platelets suspended in		Red blood cells in	
	Serum.	Salt solution.	Serum.	Salt solution.	Serum.	Salt solution.	Serum.	Salt solution.
1:1	−	+++	−	+++	−	−	−	−
1:10	−	++	−	++	−	−	−	−
1:50	−	+	−	+	−	−	−	−

+++ = complete lysis.
 ++ = incomplete lysis.
  + = slight lysis.
  − = no lysis.

It will be seen that heat destroys the lytic activity *in vitro* of the extract for blood platelets and for red blood cells, as Cole (5) and Avery and Neill (6) have already shown. However, heat does not destroy the activity of the purpura-producing constituent. Heated extract when injected into white mice causes changes in number of blood platelets similar to those following the injection of unheated extract. But the number of red blood cells alters much less. Some figures are given in Table IV.

Adsorption Experiments.—Pneumococcus extract was brought in contact with blood platelets and erythrocytes to determine the possibility of a selective adsorption. The extract was mixed with one-third its volume of washed red blood cells which had been heated for

TABLE IV.

Changes in Platelets and Erythrocytes Following the Injection of Heated Pneumococcus Extract.

Mouse IV, injected with 0.4 cc. of extract which was heated for 20 minutes at 56°C.

Time of examination.	No. of platelets.	No. of erythrocytes.	Remarks.
Before injection............	2,300,000	9,600,000	
1 day after injection......	450,000	9,000,000	Marked purpura in both ears.
2 days " "	710,000	9,100,000	" " " " "
5 " " "	1,500,000	9,200,000	

TABLE V.

The Effect of Adsorption of Pneumococcus Extracts on Platelets and Erythrocytes in Vitro.

Extract adsorbed with	Action on red cells.			Action on platelets.	
	Dilution of extract.			Dilution of extract.	
	1:1	1:5	1:10	1:1	1:5
Red cells............................	—	—	—	—	—
Platelets............................	++	+	—	+	+
White cells........................	++	+	—	+	+
Kaolin...............................	+++	++	++	+++	+
Unadsorbed........................	+++	++	++	+++	+

TABLE VI.

Changes in Platelets and Erythrocytes Following the Injection of Adsorbed Pneumococcus Extract.

Mouse , injected intraperitoneally with 0.5 cc. of extract after adsorption with erythrocytes.

Time of examination.	No. of platelets.	No. of erythrocytes.	Remarks.
Before injection.................	2,000,000	8,400,000	
1 day after injection...........	170,000	8,800,000	Purpura after 18 hrs.
3 days " "	840,000	3,100,000	

10 minutes at 70°C. Specimens of the material were kept both in the ice chest and in the incubator for $\frac{1}{2}$ hour. The extract treated in this manner was no longer lytic for either erythrocytes or platelets. Extracts similarly treated with blood platelets showed a lesser diminution in hemolytic and thrombolytic titer. (See Table V.)

The extracts submitted to treatment of the sort described were still capable of producing purpura.   Moreover, extracts from which the hemolysin had been adsorbed with red cells still produced marked anemia when injected into mice.   Table VI shows these findings.

Mixture of the extract with erythrocytes at ice box temperature resulted in a binding of the lysin without hemolysis.   The red cells upon which was fixed the lysin, when washed thoroughly and suspended in cold saline, hemolyzed completely when warmed to room temperature.

DISCUSSION.

On the whole, the behavior of the blood elements after the injection of pneumococcus extract is similar to the effects observed after the injection of other toxins.

Bunting (7) found that when saponin is given intravenously to rabbits a destruction of platelets and erythrocytes occurs and there is a lesser injury to the leucocytes.   Saponin also causes destruction of the cells in the bone marrow and in particular it causes an injury to the capillary walls resulting in extensive hemorrhages.   Bunting found that the changes in the number of platelets follows a curve independent of those of the other blood elements.

Duke (8, 9) showed that diphtheria toxin and benzene in large doses are poisonous to the bone marrow and cause a fall in the number of platelets and red cells. He thinks that the platelets themselves are also affected by these agents and that this factor contributes to the diminution in numbers.   He believes that the rapidity of the changes can be accounted for by assuming that the platelets normally are short lived bodies.

Our results seem to corroborate these views inasmuch as the evidence indicates that the diminution in the number of platelets and erythrocytes is brought about by some action other than direct lysis alone.   The lytic action is prevented *in vitro* by heating the extract and the heated extract still causes purpura.   Moreover, lysis by unheated extracts is inhibited by serum.   Adsorption of pneumococcus extract with blood platelets or red cells renders the thrombolysin inactive *in vitro*; but this procedure does not prevent the extract from producing purpura and thrombopenia in white mice.   The lesions were as extensive as those caused by unabsorbed extract.   If the diminution in the number of platelets were due to lysis alone, the number would return to normal in a much shorter time than is the case.   In instances

such as those described by Richardson (10) and Bunting in which bleeding was done to lower the number of blood platelets in rabbits, the number returned to normal in 2 or 3 days. Duke (8) has shown that when platelets are almost completely removed from the circulation in dogs by defibrinating blood and reinjecting it, the number returns to normal in 3 to 5 days. However, if toxic agents which poison the platelet-forming organs are injected, the rate of regeneration is markedly retarded.

The rapid diminution in number of platelets after the injection of pneumococcus extract seems to point to a direct action on the platelets in the circulation, while the slow rate or regeneration points to a toxic effect of the extract on the formation elements in the bone marrow. A few mice did not show purpura until 2 or 3 days after the injection. There may have been a delayed action on the circulating platelets; or, possibly a toxic action on the marrow which prevented the normal formation of platelets, until there were so few left that purpura resulted. Students of purpura know that injury to the capillary walls occurs. Intact vessels will probably not permit leakage, no matter how low the platelet count may be. Degkwitz (11) contends that the normal function of platelets is to plug up interstices between capillary endothelial cells. Should this be the case then primary injury to capillary walls need not occur.

The pathology of the purpuric lesions is being studied and search is under way for changes in the hemopoietic centers and capillary walls. Avery and Neill (unpublished work) have determined that the activity of pneumococcus extracts *in vitro* may be destroyed by heat and oxidation, but the hemolysin may still function as an antigen. Our data add evidence to the complicated nature of the hemolytic activity of pneumococcus extract.

SUMMARY.

A study has been made of the variation in number of the blood platelets, and the red and white blood cells of white mice injected with pneumococcus extract. The blood platelets were greatly diminished after the injection, the greatest decrease usually occurring after 24 hours. Purpuric lesions usually developed when the number of blood platelets became less than 500,000 per c.mm. Regeneration of

the platelets was accomplished by the 4th to the 9th day but there was an overregeneration and the return to normal did not take place until 2 weeks had elapsed.

The red cells were also greatly reduced in number, but the rate of their destruction and regeneration was somewhat slower than that of the platelets. The leucocytes were slightly if at all influenced by the pneumococcus extract.

Pneumococcus extracts were shown to be thrombolytic and hemolytic. Heat destroyed the activity of both the lysins *in vitro*. Heated extract produced purpura in mice but did not cause a severe anemia. Extracts adsorbed with either blood platelets or red blood cells showed a marked diminution in their thrombolytic and hemolytic activity *in vitro*. Such extracts, however, produced purpura as well as severe anemia and thrombopenia in mice.

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SEROLOGICAL RELATIONSHIPS OF TYPE-SPECIFIC AND DEGRADED PNEUMOCOCCI.

By HOBART A. REIMANN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

In a previous paper (1) it was shown that a strain of Pneumococcus Type I loses its specificity and virulence when subjected to certain unfavorable cultural environments. Yoshioka (2) has brought about a similar process of degradation in strains of Pneumococcus Types II and III. From these studies it is apparent that at least two general and widely different forms exist among pneumococci of the fixed types (Types I, II, and III), one comprising the virulent, type-specific, so called S strains, and the other including the avirulent, non-specific, so called R strains.

Both Griffith (3) and Yoshioka found that antisera prepared by using R strains for immunization did not agglutinate the virulent type-specific organisms and did not protect against infection caused by them. On the other hand, R strains were agglutinated by R sera and also by type-specific antipneumococcus sera regardless of type.

Type-specific pneumococci are capsulated and capable of elaborating the type-specific soluble substances which have been identified by Avery and Heidelberger as the carbohydrate constituent of the pneumococcus cell (4). Pneumococci of the R forms are not capsulated and do not elaborate the soluble substances which endow the cell with type specificity. In sequence with the recent observations of Avery and his associates (5–7), on the antigenic properties of the cellular constituents of pneumococcus, it seemed of interest to study further the immunological relationships between the so called S and R forms of pneumococci.

Methods.

Preparation of Sera.—Type I, II, and III pneumococci were repeatedly grown in broth containing dilutions of immune serum of the respective type, until upon

plating the cultures only colonies of the rough, avirulent R form appeared. From ten to twenty transfers in 1 per cent immune sera were necessary. Antipneumococcus sera were then prepared with the three type-specific S strains and were called Type I, II, and III S sera, and also with the variant R strains of their respective types which were designated Type I, II, and III R sera.

For the preparation of immune sera, the bacteria from young, actively growing cultures were suspended in salt solution and immediately killed at 56°C. to minimize the amount of autolysis or cell solution. Autolysis is undesirable since Avery and Heidelberger (7) have found that dissolution of the bacterial bodies is accompanied by dissociation of the specific antigenic complex and consequent loss of power to provoke type-specific antibodies. Rabbits were immunized according to the method of Cole and Moore (8) by the intravenous injection of pneumococci of the three specific types and of the variant strains derived from them by methods already described.

Sera were absorbed by mixing 10 per cent dilutions of the homologous serum with definite volumes of pneumococcus bodies obtained by centrifuging heated broth cultures. For instance, 0.2 cc. of the bacterial sediment was emulsified in 2 cc. of the diluted serum. The mixture was incubated for 2 hours at 37°C. and placed in the ice box overnight. The bacteria were then removed from the serum by centrifuging. R sera usually required a second exposure with one-twentieth volume of pneumococci for complete absorption.

Autolysates of the pneumococci were obtained by suspending the bacterial sediment from 150 cc. of an 8 hour dextrose broth culture in 10 cc. of salt solution and allowing the cells to disintegrate at 37°C. for 24 to 48 hours or until autolysis was nearly complete. The centrifuged supernatant fluids or autolysates were faintly opalescent. Solutions of pneumococci in bile did not give clear-cut reactions and were not used.

The pneumococcus protein and the anti pneumococcus-protein serum were prepared according to the methods of Avery and Heidelberger (4) and Avery and Morgan (5).

Considerable difficulty was encountered owing to the rapid autolysis and spontaneous agglutination of the II R and III R strains when used for agglutination tests. This was overcome to a large extent by transferring the strains eight or ten times in plain broth and finally killing by heat. Suspensions of heat-killed pneumococci were used in all agglutination tests.

Experiments with Antipneumococcus Serum.—The antipneumococcus serum (S) prepared by immunization with intact cells of the type-specific, virulent form contains predominantly an antibody reactive only with the soluble specific substance of the homologous type. However, since cultures may contain both intact cells and a variable amount of degraded cells as well as products of cell dissolution (7), and since in the material used for immunization dissociation is

TABLE I.

Agglutination and Precipitin Reactions with Type I-S Antipneumococcus Serum.

	Agglutination.				Precipitation.		
Bacterial suspensions.	Antipneumococcus serum (S) (Type I).			Normal rabbit serum. 1:10	Bacterial solutions.	Antipneumococcus serum (S) (Type I). 1:10	Control normal rabbit serum. 1:10
	1:10	1:20	1:40				
Type I-S pneumococci.........	++++	+++	+	—	Type I specific substance 1:40,000...............	++++	—
" II-S "	—	—	—	—	Type I-R autolysate...........	++	—
" I-R "	++	+++	+++	—	" II-R "	++	—
" II-R "	+	++	++	—	" III-R "	++	—
" III-R "	++	++	++	—	Pneumococcus protein solution 1:300..............	++	—
					Control salt solution..........	—	—

++++ = firm disc.
+++ = disc easily broken up.
++ = coarse agglutination or heavy precipitate.
+ = fine agglutination or light precipitate.
— = no agglutination or precipitation.

bound to occur in spite of precautions to prevent it, some of the cell protein becomes free and acting independently of the antigenic complex of the intact cell gives rise to an antiprotein immune body which is species-specific and distinct from the type-specific antibody. The usual antipneumococcus sera apparently contain both forms of antibodies.

It has been found on previous occasions that all R strains as well as the protein of pneumococci react in immune S sera regardless of type derivation. On the other hand, type-specific pneumococci (S forms) agglutinate only in their corresponding type sera. Agglutination and precipitin reactions of Type I-S antipneumococcus serum with suspensions and autolysates of various pneumococci are shown in Table I.

In Table I evidence is presented that Type I antipneumococcus serum contains specific agglutinins for homologous S strains, and precipitins for the homologous specific substance. It is not reactive with pneumococci of heterologous specific types. However, in addition to the type-specific antibodies there are present agglutinins for cells of the R forms, regardless of type derivation, and precipitins which react with autolysates of R strains and with solutions of pneumococcus protein. It would seem, therefore, that antipneumococcus serum contains the type-specific antibody together with antibodies which are common to all R strains and pneumococcus protein.

If antipneumococcus serum of this nature is absorbed with organisms of a homologous S type, it is possible to remove the type-specific antibody and leave the protein antibody intact. Table II shows the reactions obtained with a serum so treated.

It is apparent that neither the homologous S strain nor soluble specific substance derived therefrom is precipitated by the absorbed serum. The agglutinins and precipitins common to the R strains, however, remain, although somewhat diminished in titre. It is assumed that the diminution in titre is due to the absorption of some of the non-specific antibodies by some degraded forms or products of autolyzed pneumococci which appeared in the S culture during the preparation of the suspension for absorbing.

Just as it is possible to absorb the type-specific antibody from antipneumococcus serum and leave the protein antibody, so it is

likewise possible to absorb the latter and leave the former antibody practically undiminished in titre. Table III shows the serological

TABLE II.

Agglutination and Precipitin Reactions with Type I-S Antipneumococcus Serum Absorbed with Type I-S Pneumococci.

Agglutination.				Precipitation.	
Bacterial suspensions.	Absorbed serum dilutions.			Bacterial solutions.	Absorbed serum dilution. 1:10
	1:10	1:20	1:40		
Type I–S pneumococci......	−	−	−	Type I specific substance 1:40,000...............	−
" I–R "	+	+	+	Type I–R autolysate.......	+
" II–R "	+	+	−	" II–R "	+
				" III–R "	+
				Pneumococcus protein solution 1:300...........	+
				Control salt solution.......	−

TABLE III.

Agglutination and Precipitin Reactions with Type I-S Antipneumococcus Serum Absorbed with Type I-R Pneumococcus.

Agglutination.				Precipitation.	
Bacterial suspensions.	Absorbed serum dilutions.			Bacterial solutions.	Absorbed serum dilution. 1:10
	1:10	1:20	1:40		
Type I–S pneumococci.....	++++	+++	+	Type I specific substance 1:40,000...............	++++
" I–R "	−	−	−	Type I–R autolysate......	−
" II–R "	−	−	−	" II–R "	−
" III–R "	−	−	−	" III–R "	−
				Pneumococcus protein solution 1:300.........	−

behavior of a type-specific serum absorbed with an R strain of pneumococcus.

Absorption of Type I antipneumococcus serum with a strain of

the degraded R forms derived from the same type leaves the titre of the type-specific antibody unaffected. Both the agglutinins for the R forms of pneumococci and the precipitins for autolysates and pneumococcus protein are completely removed, leaving a serum reactive only with homologous Type I-S pneumococci and soluble specific substance derived from them.

The experiments cited above have been repeated and results similar to those for Type I serum have been obtained with antipneumococcus sera and the corresponding organisms of Types II and III.

Several attempts were made to produce a serum *in vivo* containing only the type-specific antibody. The usual procedure of immunization was employed with the exception that for each injection organisms were obtained directly from an infected mouse. The mouse was inoculated intraperitoneally with pneumococci and killed in 12 hours. The peritoneal cavity was washed with saline and the suspension immediately centrifuged, washed once, and heated at 56° for ½ hour. By this rapid procedure it was hoped that autolysis with its coincident dissociation of the carbohydrate and protein constituents of the bacterial antigen would be eliminated. However, after immunization of six rabbits by this method it was found that each serum contained varying traces of the non-specific protein antibody, although but slight in amount when compared with antipneumococcus sera prepared in the usual way.

Experiments with Anti-R Pneumococcus Serum.—Since no type-specific substances are elaborated by the R strains it seemed likely that sera prepared against these strains would not contain any of the specific antibodies, but only those reacting in common with all R strains. Agglutination and precipitin reactions against specific and degraded strains of pneumococci by an anti-R serum are shown in Table IV.

It is evident from Table IV that the anti-R serum does not contain any type-specific agglutinins for the S strain from which the R form was derived. Neither does it contain precipitins for the homologous soluble specific substance. Agglutinins are present, however, for both homologous and heterologous R strains. There are also precipitins for pneumococcus autolysates of all types, both S and R. The autolysates of the S strains obviously contain the free specific element

and the free protein, but the latter alone is reactive in R serum. By virtue of its common nature, the protein of each autolysate is therefore

TABLE IV.

Agglutination and Precipitin Reactions with Type I-R Serum.

Agglutination.				Precipitation.	
Bacterial suspensions	Serum dilutions.			Bacterial solutions.	Serum dilution. 1:10
	1:10	1:20	1 40		
Type I-S pneumococci......	−	−	−	Type I specific substance 1:40,000...............	−
" I-R "	++	+	+	Type I-S autolysate.......	+
" II-R "	++	+	+	" II-S "	+
" III-R "	++	+	+	" III-S "	+
				" I-R "	+
				" II-R "	+
				" III-R "	+
				Pneumococcus protein solution 1:300...........	+

TABLE V.

Agglutinanon and Precipitin Reactions with Type I-R Serum Absorbed with Type I-R Pneumococci.

Agglutination.				Precipitation.	
Bacterial suspensions.	Absorbed serum dilutions.			Bacterial solutions.	Absorbed serum dilutions. 1:10
	1:10	1:20	1:40		
Type I-R pneumococci.....	−	−	−	Type I-S autolysate........	−
" II-R "	−	−	−	" II-S "	−
" III-R "	−	−	−	" III-S "	−
				" I-R "	−
				" II-R "	−
				" III-R "	−
				Pneumococcus protein......	−

reactive. Similarly, pneumococcus protein, regardless of the form from which it is isolated, is precipitated by the R serum.

It is possible to absorb the antibodies from an R serum with an R

strain. This procedure removes the agglutinins for the R strains and the precipitins for the pneumococcus protein, leaving the serum inactive. The agglutination and precipitin reactions of a Type I-R serum absorbed with a Type I-R strain are given in Table V. The absorbed serum being inactive does not agglutinate suspensions of the R organisms or precipitate pneumococcus protein either in its purified isolated form or as it occurs in cell autolysates.

Protocols similar to Table IV were obtained by using II-R and III-R sera. Reactions similar to those shown in Table V were obtained

TABLE VI.

Agglutination and Precipitin Reactions with Anti Pneumococcus-Protein Serum.

Agglutination.				Precipitation.	
Bacterial suspensions.	Serum dilutions. Anti pneumo-coccus-protein serum.			Bacterial solutions	Serum dilution. 1:10
	1:10	1:20	1:40		
Type I–S pneumococci......	−	−	−	Type I specific substance 1:40,000.............,.......	−
" I–R "	++	+	+	Type I–S autolysate........	+
" II–R "	+	+	+	" II–S "	+
" III–R "	+	+	+	" III–S "	+
				" I–R "	+
				" II–R "	+
				" III–R "	+
				Pneumococcus protein solution 1:300...........	+

with a III-R serum absorbed with a II-R strain, and with a I-R serum absorbed with a III-R strain of pneumococci.

Experiments with Anti Pneumococcus-Protein Serum.—Because of the consistent similarity in precipitin reactions of the pneumococcus autolysates, and of the prepared pneumococcus protein in the foregoing experiments, it seemed of interest to observe the serologic behavior of a serum prepared against this protein. The agglutination and precipitin reactions of an anti pneumococcus-protein serum with S and R strains are shown in Table VI.

It is significant that the reactions in Table VI resemble those shown in Table IV. Apparently sera prepared with R strains and with pneumococcus protein have similar serologic properties. The anti-protein element in the serum obviously being common to the protein of all types of pneumococci, both S and R, causes a precipitate in all of the autolysates and agglutinates suspensions of all R strains. The intact S organisms are not affected.

The similarity between the antiprotein serum and the anti-R serum is further demonstrated by the absorption of the agglutinins in the antiprotein serum with R strains. The results are tabulated

TABLE VII.

Agglutination and Precipitin Reactions with Anti Pneumococcus-Protein Serum Absorbed with Type I-R Pneumococci.

Agglutination.				Precipitation.	
Bacterial suspensions.	Absorbed serum dilutions.			Bacterial solutions.	Absorbed serum dilution. 1:10
	1:10	1:20	1:40		
Type I-R pneumococci.....	—	—	—	Type I-S autolysate........	—
" II-R "	—	—	—	" II-S "	—
" III-R "	—	—	—	" III-S "	—
				" I-R "	—
				" II-R "	—
				" III-R "	—
				Pneumococcus protein solution................	—

in Table VII. The resemblance of Table VII to Table V is self-evident. The agglutinins for the R strains and the precipitins for the cell autolysates and pneumococcus protein have been removed, leaving an inactive serum.

DISCUSSION.

Avery and Neill (6) have shown that solutions of pneumococci have antigenic properties strikingly different from those of the intact cells themselves. When cell solutions are used for immunization, antibodies arise which react with the common protein alone. On the

other hand, when the uninjured bacterial cell is used for immunization, antibodies arise which react with the type-specific carbohydrate substance. By virtue of the common properties of the protein constituent of pneumococci, sera containing the antibody to this protein react with the protein from all types of pneumococci. Antiprotein sera obtained by immunization with dissolved pneumococci are similar in their serologic reactions to sera produced by the injection of the isolated protein.

The experiments recorded in the present paper show that sera prepared by immunization with pneumococci of degraded types (R strains) resemble in their reactions sera prepared by immunization with the cell protein alone. It would seem, therefore, that R pneumococci stimulate the formation of antibodies by virtue of the protein constituent of the cell body. Previous mention has been made of the absence of capsule formation and of the associated specific substance in the R strains. Consequently the R strains, devoid of the type-specific antigen, cannot stimulate the formation of antibodies reactive with the type-specific substances and therefore have only the antigenic properties of the isolated protein. Thus, on account of the common antigenic properties of the protein of all pneumococci it is not surprising that R sera cross-agglutinate with R strains regardless of type derivation, and precipitate the free protein in autolysates of S strains.

The results on the whole substantiate the deductions of Avery and Heidelberger (7) regarding the relationship of the carbohydrate and protein constituents of the pneumococcus cell.

Assuming that the loss in type specificity of an S strain after degradation to the R form is due to a loss in the capacity to elaborate the type-specific carbohydrate substance associated with the capsule, and that the R form is merely an S cell minus this factor, it is possible to interpret the immune mechanism involved according to the views of these authors. Applying these conclusions to the results of their study it is only necessary to substitute in their scheme (7, Fig. 1) the symbol for protein (P) by R representing an R cell. The type-specific cell would then be an S-R combination and the R cell, R alone.

The confusing cross-reactions occasionally encountered in typing

pneumococci with the usual diagnostic sera, and the cross-reactions occasionally noted in S cultures containing unusual numbers of degraded pneumococci, are thus in part explained.

CONCLUSIONS.

1. Immune sera prepared with the degraded or variant forms of pneumococci (R strains) are similar in their reactions to sera prepared with the protein or cell solutions of pneumococci. They contain antibodies reactive with the protein of all types of pneumococci, but no antibodies reactive with the type-specific substances.

2. Pneumococci of the variant or R form, regardless of type derivation, are serologically identical and have the antigenic characteristics of pneumococcus protein. They evoke the species-specific and not the type-specific antibodies.

3. Antipneumococcus sera obtained by immunization with S strains may contain species-specific antibodies in addition to those which are type-specific. Each kind of antibody can be removed separately from these sera by selective absorption with the R or S strains of pneumococci.

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NUCLEAR INCLUSIONS IN THE TESTICLES OF MONKEYS INJECTED WITH THE TISSUE OF HUMAN VARICELLA LESIONS.

BY THOMAS M. RIVERS, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

PLATES 5 AND 6.

Some reasons for studying chicken-pox together with a review of the literature concerning the attempted experimental production of the disease in animals have been given in a previous paper (1). Recently several papers have appeared in support of the view that there is a close relation between chicken-pox and herpes zoster (2) on the one hand and between herpes zoster and herpes simplex (3) on the other. From Doerr's (4) comprehensive review of the literature concerning these diseases one concludes that the proof of the identity of the three or of any two of them is inadequate. Furthermore, it is doubtful if more than one, herpes simplex, has been experimentally produced in animals. Therefore, to solve many of the problems arising in connection with these diseases, it is desirable to establish the other two in animals and this I have attempted to do with varicella.

Early in the work attempts were made to infect monkeys with the virus of chicken-pox. Only Indian macaques were employed and no positive results were obtained. Meantime a filterable virus was discovered during attempts to transmit varicella to rabbits (1). At first this virus (Virus III) was thought to be the etiological agent of chicken-pox. Subsequent work (5), however, in my laboratory and also in Swift's (6) disclosed the fact that Virus III is not the cause of varicella but is indigenous to rabbits. Following this disclosure, the use of monkeys in the experimental study of chicken-pox was resumed and it is with some results of the work that the present paper deals.

Methods and Materials.

Monkeys Employed.—It is well known that species of monkeys vary in susceptibility to certain diseases. Therefore, in addition to Indian macaques (*Macacus rhesus*), African vervets (*Cercopithecus lalandi*) and South American ringtail monkeys (genus *Cebus*) and marmosets (*Hapale jacchus*) were employed. 10 monkeys were inoculated, all of which were young; in none, with the exception of the marmoset, had spermatogenesis been established.

Other Animals.—In addition to the monkeys, 2 rabbits, 1 guinea pig, 1 white rat, and 3 chickens were used.

Inoculations.—Blood, vesicle fluid, and emulsified papules and vesicles collected from patients with varicella, usually during the first 48 hours of the disease, were used for inoculation. Pieces of skin from a normal volunteer were used as a control. The blood was collected and injected before clotting occurred. The fluid from vesicles, to which were added scrapings from the floor of the lesions, was collected in sterile capillary pipettes. The papules and vesicles were excised under aseptic conditions and emulsified by grinding in a mortar moistened with Locke's solution. Sand was not used. The emulsified material was taken up in 0.5–1.0 cc. of Locke's solution and injected by means of a tuberculin syringe. More than 15–20 minutes never elapsed between the collection of the material from the patients and its inoculation into animals. All monkeys were inoculated intratesticularly (0.2–0.25 cc.). In addition to this route, some were inoculated intravenously, intracutaneously (0.1 cc.), and in the inguinal lymph nodes (0.2 cc.). The rabbits, guinea pig, and white rat were inoculated intratesticularly; the chickens in the wattles.

Examination of Animals and Tissues.—Daily examinations of the animals were made for 3 weeks following the inoculations. Tissues, removed under ether anesthesia for histological studies, were fixed in Zenker's fluid with 5 per cent acetic acid, sectioned, and stained with methylene blue and eosin. In addition to a study of the general character of the lesions, a very careful search for eosin-staining nuclear inclusions was made in numerous sections of all the tissues. Details concerning the tinctorial reactions of these inclusions are given by Tyzzer (7), Lipschütz (8), Goodpasture (9), and others.

EXPERIMENTAL.

In the experiments to be reported, the majority of the monkeys, 7 of 10, were vervets. The reason for this will become obvious furthur on and the chief interest in these monkeys centers around the results obtained by means of intratesticular inoculation of emulsified vesicles and papules. In connection with this phase of the work, 14 experiments were performed; and in view of the results obtained it seems advisable to give a detailed account of each.

Experiment 1.—Monkey A; vervet. November 12, 1924. 10 cc. of blood collected from varicella patient, Case 1, 36 hours after the appearance of the eruption, was injected intravenously into Monkey A. Fluid from 30 vesicles was also collected at the same time and injected intradermally in left eyelid, in left and right thighs, and in right side of abdominal wall. While under observation the animal showed no manifestations suggestive of chicken-pox. The temperature ranged from 101.7° to 102.9°F. No tissue was removed for histological study.

Although Monkey A showed no visible specific reaction, it is possible that he became infected and that an immunity to chicken-pox was established. 5 months later he was used for intratesticular inoculation (Experiment 11).

Experiment 2.—Monkey B; vervet. Chicken A. March 5, 1925. 2 papules were removed from varicella patient, Case 2, 18 hours after the appearance of the eruption, emulsified, taken up in 1.0 cc. of Locke's solution, and injected as follows: Monkey B, 0.2 cc. in each testicle, 0.2 cc. in left inguinal lymph node, and 0.1 cc. intradermally; Chicken A, 0.1 cc. in right wattle.

Monkey B.—While under observation the animal showed no gross significant reaction. The temperature ranged from 101.6° to 103.5°F., reaching its highest mark 9 days after the inoculation. A skin nodule, removed on the 5th day, showed a central necrosis surrounded by polymorphonuclear and mononuclear cells. No nuclear inclusions were seen. While the right testicle, removed on the 5th day, showed upon gross examination evidences of injury, nothing of a specific nature was observed. After fixation the testicle was divided into three parts and sections were made of each block. Block 1 showed definite evidences of injury to the tubules and interstitial tissue with an infiltration of mononuclear and polymorphonuclear cells. No nuclear inclusions were found. In Block 2 the reaction was of the same general character as that observed in Block 1, with the exception that it was fairly well localized to a narrow streak running through the section. At one point (Fig. 1) in the damaged interstitial tissue numerous cells, which looked like endothelial leucocytes, were swollen and had within their nuclei typical eosin-staining inclusions (Fig. 6). The inclusions were found in all sections from this block. Block 3: Very little evidence of injury and no nuclear inclusions were observed. The right inguinal lymph node, removed on the 10th day, showed necrotic areas without nuclear inclusions. In the visceral tunic of the left testicle, removed on the 10th day, were found about 20 minute discrete reddish nodules. After fixation the testicle was divided into three parts and sections were made from each block. Blocks 1, 2, and 3: The visceral tunic was thickened and more cellular than usual. In addition to the general thickening, definite localized lesions, made up for the most part of mononuclear cells, were found. The tubules showed very little damage but a few localized lesions were seen in the interstitial

tissue. All the lesions in this testicle were healing and no nuclear inclusions were seen.

Chicken A.—The nodule in the wattle was removed on the 12th day. Upon microscopic examination a central necrosis surrounded by mononuclear cells was found. No nuclear inclusions were observed.

In Experiment 2, typical pink-staining nuclear inclusions were found in the vervet's testicle removed 5 days after inoculation with emulsified varicella papules. None were found, however, in the testicle removed after 10 days.

Experiment 3.—Monkey C; vervet. March 12, 1925. Fluid and scrapings from 20 vesicles were collected from varicella patient, Case 3, 36 hours after the appearance of the rash, mixed with 1.0 cc. of Locke's solution, and injected into Monkey C as follows: 0.25 cc. in each testicle, 0.25 cc. in right inguinal lymph node, and 0.1 cc. intradermally. While under observation the animal showed no significant reaction. The temperature ranged from 101.9° to 103.5°F., reaching its highest point on the 3rd and 13th days after the inoculation. The left testicle was removed on the 11th day, the right on the 13th, and upon gross examination neither showed anything of a specific nature. Each testicle was divided into three parts after fixation and sections were made from each block. The visceral tunic of both was thickened and here and there discrete lesions, characterized by an infiltration of mononuclear cells, were seen. Just beneath the visceral tunic, collections of mononuclear cells were also found. Many of the tubules were damaged and in certain areas of the interstitial tissue there were collections of mononuclear cells. No nuclear inclusions were observed.

The left and right testicle of Monkey C were removed on the 11th and 13th day respectively after inoculation and showed healing lesions in which no nuclear inclusions were seen.

Experiment 4.—Monkey D; vervet. March 23, 1925. 2 skin lesions were removed from varicella patient, Case 4, 48 hours after the appearance of the rash, emulsified, taken up in 0.75 cc. of Locke's solution, and injected into Monkey D as follows: 0.2 cc. in each testicle, 0.1 cc. in right inguinal lymph node, and 0.1 cc. in the skin on the left side of the abdomen. No significant reactions were observed. The temperature ranged from 101.5° to 103.5°F., reaching the highest point on the 4th day after inoculation. The skin nodule and right inguinal lymph node, removed on the 5th day, showed no significant reaction and no nuclear inclusions. The right testicle, removed on the 5th day, upon gross examination showed nothing characteristic. After fixation it was divided into three parts and sections were made from each block. Block 1: The tunic was only slightly damaged. The testicular tissue showed marked evidences of injury with an infiltration of

mononuclear and polymorphonuclear cells in the interstitial tissue. No nuclear inclusions were found. Block 2: The picture was of the same general character as that seen in Block 1. In one area, however, slightly removed from the major portion of the reaction and located just beneath the tunic there was found a discrete lesion (Fig. 2) characterized by hemorrhage and an infiltration of mononuclear cells. Although a few tubules were injured, the principal part of the lesion was in the interstitial tissue and numerous endothelial leucocytes were swollen and contained typical eosin-staining nuclear inclusions (Fig. 3). Block 3: Very little reaction was seen except at one point where there was a definite collection of mononuclear cells. Careful examinations of the left testicle, removed on the 7th day, showed that the inoculum had not entered the gland. No nuclear inclusions were found.

Although no nuclear inclusions were found in the skin and the lymph node removed from Monkey D on the 5th day after inoculation, typical ones were found in the right testicle removed on the same day.

Experiment 5.—Monkey E; vervet. March 24, 1925. 5 lesions, vesicles and papules, were removed from varicella patients—2 from Case 5, 2 from Case 6, and 1 from Case 7—within 48 hours after the appearance of the rash, and ground up together. The emulsified material was taken up in 0.75 cc. of Locke's solution and injected into Monkey E as follows: 0.25 cc. in each testicle, and 0.1 cc. in each of two places in the skin of the abdominal wall. No significant reactions appeared in the animal while under observation. The temperature ranged from 101.6° to 103°F., reaching the highest point on the 1st and 6th days after inoculation. The skin nodule, removed on the 6th day, showed a thickening of the epidermis, and a localized necrosis in the corium surrounded by polymorphonuclear and mononuclear cells. None of the cells contained nuclear inclusions. The left testicle, which was removed on the 6th day, looked as though it had been considerably injured by the inoculation. After fixation it was divided into three parts and sections were made from each block. Blocks 1 and 2: Necrosis of some of the tubules and collections of polymorphonuclear and mononuclear cells in the interstitial tissue were found. Eosin-staining nuclear inclusions were seen in cells of the tubules and in endothelial leucocytes of the interstitial tissue. Block 3: Lesions of the same general character as those seen in Blocks 1 and 2 were encountered. Tubules having cells which contained nuclear inclusions were much more numerous, however. Many of the tubules with swollen cells and nuclear inclusions resembled chicken-pox vesicles (Figs. 4 and 7). Numerous nuclear inclusions were also found in endothelial leucocytes in the interstitial tissue. The right testicle, removed on the 8th day, was also considerably damaged by the inoculation. After fixation it was divided into three parts and sections were made from each block. Extensive damage to the visceral tunic and testicular tissue

with necrosis and infiltration of polymorphonuclear and mononuclear cells was seen in all. No nuclear inclusions were found, however.

Monkey E received the largest inoculum of any animal in the series, getting in all the emulsified tissue of 5 varicella lesions in concentrated form. In this animal for the first and only time I observed nuclear inclusions in glandular cells of the testicle. These eosin-staining bodies were typical of the nuclear inclusions seen in several virus diseases. It is of importance to note that the testicle removed on the 6th day contained many nuclear inclusions while the one removed on the 8th day, although just as badly damaged as the other, showed none. From this it would seem that 8 days is too long a time to wait before castration if one is looking for inclusions.

Experiment 6.—Monkey F; vervet. March 26, 1925. From each of two varicella patients, Cases 8 and 9, a papule and a vesicle were removed within 48 hours after the appearance of the eruption, emulsified, taken up in 1.0 cc. of Locke's solution, and injected into Monkey F as follows: 0.25 cc. in each testicle, and 0.1 cc. in each of three places in the skin over the abdomen. No significant reaction appeared in the animal while under observation. The temperature ranged between 102° and 103.8°F., reaching the highest point 24 hours after inoculation. A skin nodule was removed on the 5th and the 7th days. Upon gross and microscopic examination these showed nothing of a specific nature. The left testicle, removed on the 5th day, was nodular, and hemorrhagic spots were seen in the tunic. After fixation the testicle was divided into three parts and sections were made from each block. Block 1: Considerable damage to the tunic and the testicular tissue had resulted from the inoculation. There was nothing specific in the reaction as a whole. In one area, however, numerous endothelial leucocytes were swollen and contained eosin-staining nuclear inclusions. Block 2: The reaction was in general similar to that in Block 1 but there were no nuclear inclusions. Block 3: The reaction was of the same general character as that seen in Blocks 1 and 2. In one area in the interstitial tissue somewhat removed from the point of maximal damage, there was a collection of endothelial leucocytes some of which contained typical pink nuclear inclusions. The right testicle, removed on the 7th day, did not seem to be damaged. Sections from three blocks showed that the inoculum had not entered the gland. The testicular tissue was normal and no inclusions were found. Sections of the epididymis showed considerable damage with collections of polymorphonuclear and mononuclear cells. No inclusions were found, however.

Nuclear inclusions were found in two of three blocks from the left testicle of Vervet F removed 5 days after inoculation. No inclu-

sions were found in pieces of skin removed 5 and 7 days following the inoculation.

Experiment 7.—Chickens B and C.   March 27, 1925.   2 recently formed vesicles were removed from varicella patient, Case 10, within the first 48 hours of the disease, emulsified, and taken up in 1.0 cc. of Locke's solution.   0.2 cc. of the emulsion was injected into each wattle of two chickens, B and C.   A wattle was removed on the 4th, 5th, and 7th days after inoculation.   Nothing specific was observed upon gross or upon microscopic examination.   No nuclear inclusions were seen.

Experiment 8.—Monkey G; ringtail.   April 2, 1925.   From varicella patient, Case 11, 3 lesions, 1 papule and 2 vesicles, were removed within 36 hours after the onset of the disease, emulsified, taken up in 0.75 cc. of Locke's solution, and injected into Monkey G as follows: 0.2 cc. in each testicle and 0.1 cc. in each of 2 areas in the skin of the abdominal wall.   While under observation the animal showed nothing of a specific nature.   The temperature ranged from 102° to 103.5°F., reaching the highest point 24 hours after inoculation.   A skin nodule and both testicles were removed on the 5th day.   Each testicle was divided into three parts and sections were made from each block.   In sections of the skin the epidermis was thickened and the corium was necrotic in places and contained many mononuclear and polymorphonuclear cells.   No inclusions were seen.   Both testicles had been injured by the inoculation and showed necrosis, hemorrhage, and collections of mononuclear and polymorphonuclear cells.   No nuclear inclusions were found.

Experiment 9.—Guinea Pig A.   April 3, 1925.   A papule and a vesicle were removed from varicella patient, Case 11, on the 3rd day of the disease, emulsified, taken up in 0.5 cc. of Locke's solution, and injected into the testicles of Guinea Pig A.   Nothing of a specific nature was observed in the animal while under observation.   The temperature ranged from 100.8° to 102.2°F.   Both testicles, removed on the 5th day, were divided into three parts each after fixation, and sections were made from each block.   Both testicles had been damaged by the inoculation.   There were areas of necrosis surrounded by hemorrhage and collections of mononuclear and polymorphonuclear cells.   No nuclear inclusions were seen.

Experiment 10.—Rabbit A.   April 5, 1925.   A papule and a vesicle were removed from varicella patient, Case 12, 36 hours after the appearance of the rash, emulsified, taken up in 0.5 cc. of Locke's solution, and injected into the left testicle of Rabbit A.   Nothing specific was noted in the animal while under observation. Its temperature ranged from 102° to 103.5°F., reaching the highest point 4 days after the inoculation.   Both testicles were removed on the 5th day.   After fixation the right was divided into four parts and the left into five.   Sections were made from each block.   The right uninoculated testicle proved normal.   The left testicle had been considerably damaged by the inoculation but no nuclear inclusions were found.

Experiment 11.—Monkey A, vervet; Monkey H, macaque; White Rat A. April 7, 1925. 24 hours after the appearance of the eruption, 5 lesions were removed from 2 varicella patients—1 vesicle and 2 papules from Case 13 and 2 papules from Case 14. The material was emulsified, taken up in 1.0 cc. of Locke's solution, and injected, into 3 animals as follows: 0.25 cc. in the right testicle of Monkey A; 0.15 cc. in each testicle and 0.1 cc. in the skin of the abdominal wall of Monkey H; and 0.1 cc. in each testicle of White Rat A.

Monkey A, Vervet.—The right testicle, removed on the 6th day, was divided into three parts after fixation. Sections made from each block showed that considerable damage to the tubules and interstitial tissue had resulted from the inoculation. No nuclear inclusions were found.

Monkey H, Macaque.—The left and right testicle, removed on the 4th and 6th day respectively, were divided and sectioned in the usual manner. Considerable damage to the tubules and interstitial tissue had resulted from the inoculation but no nuclear inclusions were found.

White Rat A.—Both testicles, removed on the 6th day, were divided and sectioned in the usual manner. Although considerable damage to the tubules and interstitial tissue had resulted from the inoculation, no nuclear inclusions were seen.

Monkey A, vervet, had already been used in Experiment 1 and at the time of inoculation might have been immune to varicella because of the previous injection. At any rate no nuclear inclusions were found in the right testicle 6 days after inoculation.

Experiment 12.—Monkey I; marmoset. April 8, 1925. 2 vesicles and 1 papule were removed from varicella patient, Case 15, within 48 hours after the appearance of the rash, emulsified, taken up in 0.5 cc. of Locke's solution, and injected into Monkey I, 0.15 cc. in each testicle. Both testicles were removed on the 5th day and after fixation each one was divided into three parts. Sections from each block showed the damage to the tubules and interstitial tissue which is an inevitable result of the inoculation, but no nuclear inclusions were found.

Experiment 13.—Rabbit B. April 9, 1925. 1 vesicle and 1 papule were removed from varicella patient, Case 16, about 36 hours after the appearance of the eruption. The material was emulsified, taken up in 0.75 cc. of Locke's solution, and injected into the right testicle of Rabbit B. This testicle was removed on the 4th day and after fixation was divided into seven parts. Sections were made from each block. Considerable damage to the tubules and interstitial tissue had resulted from the inoculation, but no nuclear inclusions were found.

Experiment 14.—Monkey J; vervet. April 11, 1925. 2 pieces of skin were removed from a normal adult volunteer, emulsified, taken up in 0.4 cc. of Locke's solution, and injected into Monkey J, 0.2 cc. in each testicle. The temperature of the animal ranged from 101.8° to 104°F., reaching the highest point 48 hours

TABLE I.

Occurrence of the Nuclear Inclusions.

Experimental animal.	Inoculum.	Tissues examined histologically.	No. of days after inoculation.	Nuclear inclusions.
Vervet A (immune ?).	Emulsified lesions.	Right testicle.	6	—
Vervet B	" "	Skin.	5	—
		Right testicle.	5	+
		Left "	10	—
		Inguinal lymph node.	10	—
" C	Vesicle fluid and scrapings.	Left testicle.	11	—
		Right "	13	—
" D	Emulsified lesions.	Skin.	5	—
		Inguinal lymph node.	5	—
		Right testicle.	5	+
" E	" "	Skin.	6	—
		Left testicle.	6	+
		Right "	8	—
" F	" "	Skin.	5	—
		"	7	—
		Left testicle.	5	+
" J	Skin from normal volunteer.	Right "	5	—
Ringtail G	Emulsified lesions.	Skin.	5	—
		Right testicle.	5	—
		Left "	5	—
Macaque H	" "	Right "	4	—
		Left "	6	—
Marmoset I	" "	Right "	5	—
		Left "	5	—
Rabbit A	" "	Left "	5	—
" B	" "	Right "	4	—
Guinea Pig A	" "	" "	5	—
		Left "	5	—

TABLE I—*Concluded.*

Experimental animal.	Inoculum.	Tissues examined histologically.	No. of days after inoculation.	Nuclear inclusions.
White Rat A	Emulsified lesions.	Right testicle.	6	—
		Left "	6	—
Chicken A	" "	Right wattle.	12	—
" B	" "	" "	4	—
		Left "	5	—
" C	" "	Right "	7	—

+ indicates presence of eosin-staining nuclear inclusions.
— " absence " " " "

Testicles into which the inoculum did not enter are not indicated in this table.

after inoculation. The right testicle, removed on the 5th day, was divided into three parts after fixation. Sections from each block showed that damage to the tubules and interstitial tissue had resulted from the inoculation. This damage was characterized by necrosis, hemorrhage, and collections of mononuclear and polymorphonuclear cells. No nuclear inclusions were found. The left testicle, removed on the 7th day, was normal since the inoculum had not entered the gland. No inclusions were found.

A summary of the experiments with special reference to the presence of eosin-staining nuclear inclusions in the inoculated tissues is given in Table I.

DISCUSSION.

A number of diseases are characterized by fever, papules and vesicles showing some destruction of the skin and infiltration of it with polymorphonuclear and mononuclear cells. In some of them, *e.g.* chicken-pox, herpes simplex, and herpes zoster, the gross and microscopic appearance of the skin lesions are so similar as to be almost identical. In addition to this, eosin-staining nuclear inclusions (Fig. 5) in the epidermal cells and endothelial leucocytes are a most characteristic feature. Furthermore, tissues experimentally damaged by the virus of herpes simplex regularly show acidophilic nuclear bodies (10). Similar inclusions are also found in lesions produced by Virus III (11) which is indigenous to rabbits.

The eosin-staining nuclear inclusions are so consistently found under certain conditions that to many workers they appear extremely significant. Some consider them merely as products of degeneration, but others believe that they are the virus itself, while yet others think of them as virus surrounded by a mantle of altered nuclear material. As yet their nature has not been definitely determined. This fact, however, does not lessen their significance for experimental work. I have never found typical, eosin-staining nuclear inclusions save in virus diseases, and this has been the experience of other workers (12). In view of this fact there is reason to believe that infection with a virus has taken place when such inclusions are found (10). To find them is not always easy, especially in experimental animals, and a careful search should be made before a negative result is recorded. Furthermore, it must be kept in mind that there is an optimum time (9) following the inoculation for the finding of the inclusions after which they disappear rapidly.

For the 14 experiments reported in this paper, material from 16 typical cases of chicken-pox was used. The wide derivation of the material lessens the chances of an incorrect diagnosis affecting the results. The nuclear inclusions were found only in the testicles of 4 vervets,—which had been inoculated with material from different sources,—and not in other inoculated tissues of the same animals, nor anywhere in the other experimental animals (Table I). Furthermore, they were not found in the testicles of a vervet inoculated with normal skin, in the testicle of a vervet inoculated with emulsified varicella lesions but possibly immunized by a previous injection, nor in a vervet's testicles removed 11 and 13 days after inoculation with vesicle fluid and scrapings. The testicles of the 4 vervets in which inclusions were found were removed 5 and 6 days after inoculation while those removed after 8 and 10 days, although inoculated with the same material and as severely traumatized as the others, contained no inclusions. It is of importance that the 4 vervets in which positive results were obtained came from at least two sources and were not in contact with one another at any time. In the light of what is known concerning eosin-staining nuclear inclusions and in view of the conditions under which the experiments reported in this paper were performed, it seems reasonable to conclude that the acido-

philic nuclear inclusions in the 4 vervets' testicles were manifestations of the presence of a virus. The nature of the virus and the possibility of transmitting it through a series of monkeys remain to be determined.

CONCLUSIONS.

Eosin-staining nuclear inclusions resembling those deemed characteristic of a certain well known group of filterable viruses, amongst which is varicella, were found in vervets' testicles inoculated with emulsified tissue of human varicella lesions.

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EXPLANATION OF PLATES.

PLATE 5.

FIG. 1. Lesion in right testicle of Vervet B following the injection of emulsified varicella material. *A* indicates area from which Fig. 6 was drawn. × 135.

FIG. 2. Discrete lesion in the right testicle of Vervet D 5 days after inoculation with varicella material. *A* indicates area from which photograph in Fig. 3 was taken. × 135.

FIG. 3. Nuclear inclusions indicated by *B* in the lesion shown in Fig. 2. × 1000.

FIG. 4. Lesion in left testicle of Vervet E following the injection of emulsified varicella material. *A* indicates area from which Fig. 7 was drawn. × 135.

PLATE 6.

FIG. 5. Nuclear inclusions in epidermal cells of human varicella lesion.   Eosin-methylene blue.   × 600.

FIG. 6. Nuclear inclusions in endothelial leucocytes of right testicle of Vervet B. Eosin-methylene blue.   × 600   × 1200.

FIG. 7. Nuclear inclusions in gland cells of the left testicle of Vervet E.   Eosin-methylene blue.   × 600.   × 1200.
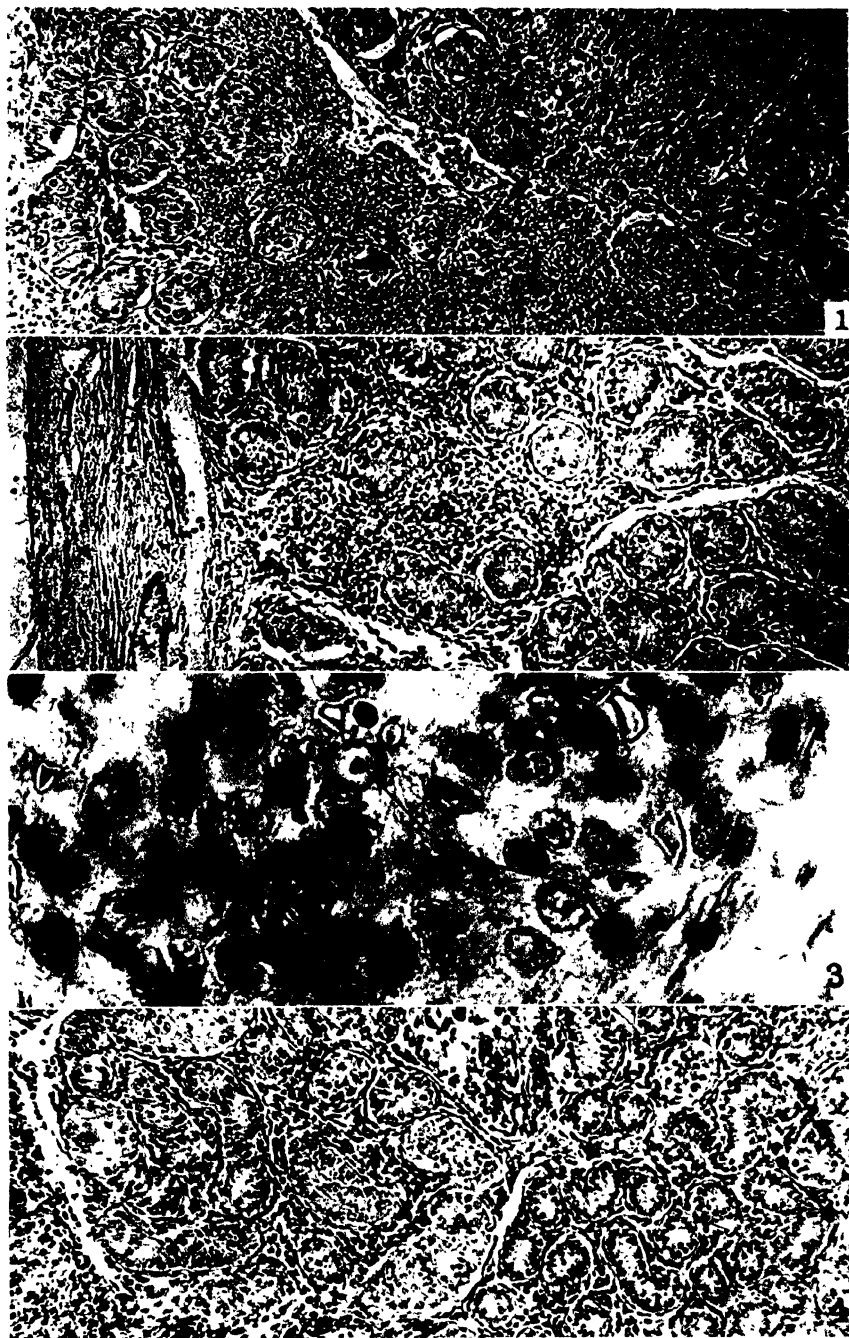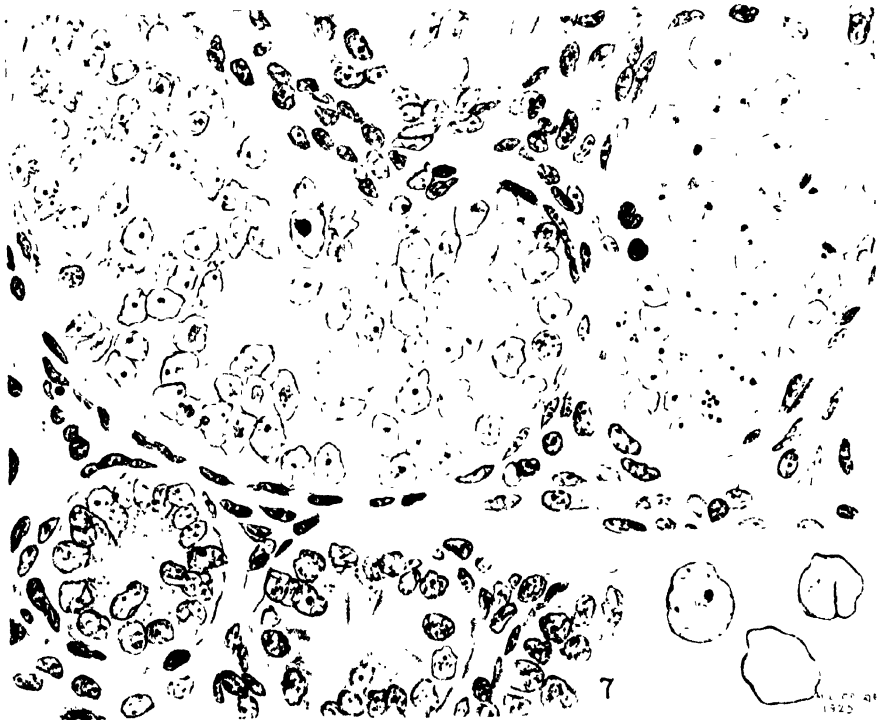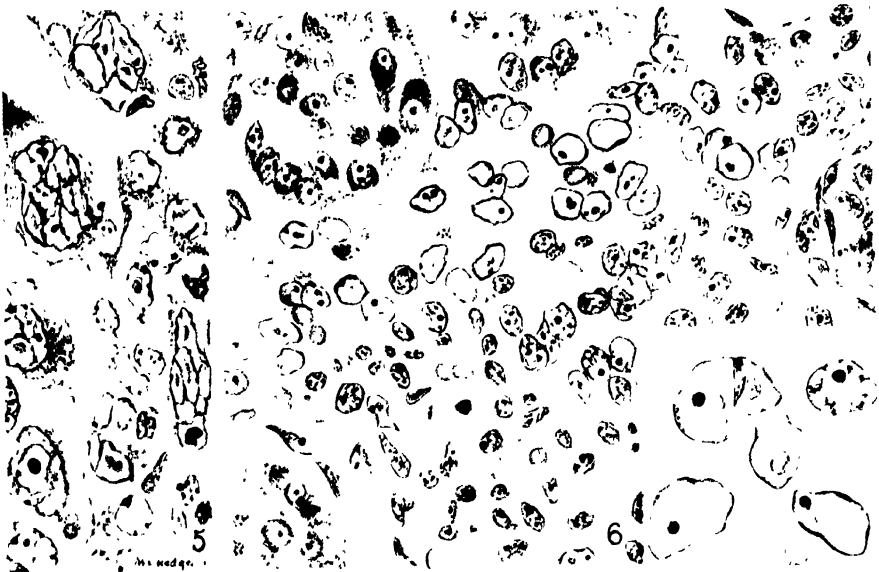
THE DETERMINATION OF GASES IN BLOOD AND OTHER SOLUTIONS BY VACUUM EXTRACTION AND MANOMETRIC MEASUREMENT.

III. GASOMETRIC DETERMINATION OF METHEMOGLOBIN.

BY DONALD D. VAN SLYKE.

(From the Hospital of The Rockefeller Institute for Medical Research, New York.)

Barcroft and Müller (1911) apparently proposed the first method for quantitative determination of methemoglobin. It was based on the fact that methemoglobin does not form a reversible compound with oxygen. Consequently the difference between the total hemoglobin (determined colorimetrically) and the portion of hemoglobin capable of combining with oxygen indicated the methemoglobin. Subsequent methods have been based on the same principles. The improvements have been in the methods for determining the total hemoglobin and the active hemoglobin, as we may call the hemoglobin capable of combining with oxygen.

In the recent literature three accurate methods have appeared.

Stadie (1920) changes both methemoglobin and active hemoglobin to cyanhemoglobin, and determines the total hemoglobin colorimetrically as cyanhemoglobin.

Nicloux (1924) by the simultaneous action of sodium hydrosulfite and carbon monoxide changes both methemoglobin and active hemoglobin to carboxyhemoglobin, which he estimates from the amount of CO held in combination. In accordance with Conant's elucidation of the relationship between methemoglobin and active hemoglobin, the reaction of methemoglobin may be schematically represented as

HbOH	+ H $\longrightarrow$	Hb	+ CO $\longrightarrow$ HbCO
Methemoglobin.	Reduction by hydrosulfite.	Reduced hemoglobin.	Carboxy-hemoglobin.
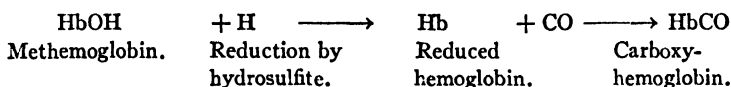
The inactive methemoglobin is reduced by hydrosulfite to reduced

hemoglobin, which is capable of combining with O_2 or CO.   Nicloux accordingly estimated the methemoglobin from the difference in CO-combining capacity before and after treatment with hydrosulfite.

Conant and Fieser (1925) also reduce the methemoglobin to reduced hemoglobin.   They make their analyses, however, by determining the oxygen-combining capacity, instead of the equivalent CO capacity. As reducing agent they found that they could not use hydrosulfite because some of its products catalyzed the regeneration of methemoglobin which began as soon as oxygen was admitted to the reduced solution.   They obtained excellent results, however, when they performed the reduction with beta-anthrahydroquinone sulfonate, and limited the subsequent period of oxygenation to 10 or 15' seconds before drawing the sample for determination of combined oxygen. Even when beta-anthrahydroquinone sulfonate was used as reducing agent, longer exposure to oxygen resulted in formation of appreciable amounts of methemoglobin, and consequently low results for total hemoglobin estimated from the oxygen capacity.

As Conant and Fieser point out, their entirely gasometric technique has an advantage over the partially colorimetric method of Stadie, in that the presence of hematin or other colored products does not interfere with the former.   .

Nicloux's carbon monoxide gasometric method has the same advantages.   It also has an advantage over the technique of Conant and Fieser in that the possibility of regeneration of methemoglobin is avoided by the use of CO instead of O_2 for saturation of the reduced hemoglobin.

In the technique described in the present paper, we have adapted Nicloux's method to use with the manometric blood gas apparatus, with which rapid and accurate CO determinations have been described by Van Slyke and Neill (1924), and by Harrington and Van Slyke (1924).

Method for Determination of Methemoglobin.

The reagents used are the following:

Acid Ferricyanide Solution.—Described by Van Slyke and Neill.[1]

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[1] Van Slyke and Neill (1924), p. 563.

Air-Free 1 N Sodium Hydroxide Solution.—Described by Van Slyke and Neill.[2]

Carbon Monoxide Gas.—This may be prepared by dropping concentrated sulfuric acid upon crystals of oxalic acid or into anhydrous formic acid, and warming with a burner, the evolved gas being collected in a gasometer. Oxalic acid yields per gm. about 275 cc. each of CO_2 and CO; the gases must accordingly be washed with alkali to remove CO_2. Formic acid yields per cc. about 500 cc. of pure CO.

Nicloux's Ammoniacal Solution of Sodium Hydrosulfite.—Place in a 100 cc. beaker 2 gm. of pulverized $Na_2S_2O_4$. Mix in another beaker 50 cc. of water and 1 cc. of concentrated ammonia solution. Pour the dilute ammonia solution onto the hydrosulfite, and cover the solution at once with a layer of paraffin oil about 1 cm. deep to prevent oxidation by air. Dissolve the hydrosulfite by stirring for a few seconds with a rod. Commercial hydrosulfite usually contains some insoluble impurity; consequently one does not attempt to stir until complete solution is attained.

Procedure of Analysis.—Place in a separatory funnel, preferably of cylindrical rather than conical shape, either 5 or 10 cc. of blood, accurately measured, and $\frac{1}{5}$ as great a volume, also accurately measured, of the freshly prepared ammoniacal hydrosulfite solution, making blood solution A. Smaller amounts of blood may be used if micro determinations of the CO are made. The separatory funnel should be large enough so that the blood will spread over the walls in a thin layer when rotated. A 100 cc. funnel is convenient for 5 cc. of blood, which is used when 1 cc. samples are to be taken for CO determination.

The air in the funnel is immediately replaced by CO at atmospheric pressure. This may be accomplished by passing through the funnel several times the volume of CO that would fill it. Somewhat more economically, with respect to CO consumption, it may be accomplished by alternately evacuating the funnel and refilling it with CO three times.

In a second similar funnel are placed the same volume of blood, $\frac{1}{5}$ volume of water (instead of hydrosulfite solution), and an atmosphere of CO, in the same manner (blood solution B).

The vessels are now rotated, either by hand or by a mechanical rotator like that of Stadie (1921), for 15 minutes or more. Then samples of 0.2, 1.0, or 2.0 cc., according to the amount of blood available, are drawn from the funnel into pipettes and used for carbon

[2] Van Slyke and Neill (1924), p. 534.

monoxide determinations.   It does not matter how long the blood is left in the vessels with the CO atmosphere, but the samples for analysis should be drawn into pipettes, especially in the case of the blood treated with hydrosulfite, as soon as the funnel is opened to the air.

The carbon monoxide determinations are carried out as described by Van Slyke and Neill,[3] with the simplification that absorption of O_2 from the extracted blood gases is here unnecessary, since all the O_2 has been replaced by CO.   The CO_2 is absorbed with the 1 N air-free sodium hydroxide, and p_1 is measured on the manometer.   The gas is then ejected from the apparatus, and p_2 is measured.   The CO pressure is

$$P_{CO} = p_1 - p_2.$$

Calculation.—The carbon monoxide contents are calculated by means of Table II or III of Van Slyke and Neill.   In order to correct for the dilution with $\frac{1}{5}$ volume of hydrosulfite solution or water each result is multiplied by $\frac{6}{5}$.   The methemoglobin is calculated as follows, in terms of O_2 or CO capacity:

$$\text{Methemoglobin} = (\text{CO in A}) - (\text{CO in B}).$$

The total hemoglobin content may be estimated as

$$\text{Total Hb} = (\text{total CO in A}) - (\text{dissolved CO}).$$

The hemoglobin values are thus obtained in terms of volume per cent or millimols per liter of oxygen (or CO) capacity.

The estimation of the "dissolved CO" is somewhat uncertain because of the solubility coefficient of CO in blood has not been accurately ascertained by direct determinations.   These were attempted by Hüfner (1894), but satisfactory results were prevented by the difficulty in differentiating between physically dissolved and combined CO.   However, we have found that sufficiently close results are obtained if the solubility is assumed to be proportional to the water content of the diluted blood, or 85 per cent of the solubility in pure water.   Thus estimated the dissolved CO taken up when the saturation is performed at a barometric pressure of 760 mm. is in terms of volumes per cent, 2.16 at 15°, 1.95 at 20°, 1.82 at 25°, and 1.71 at 30°.

[3] Van Slyke and Neill (1924), p. 562.

The values in terms of millimolar concentrations are 0.99 at 15°, 0.87 at 20°, 0.8 at 25°, and 0.76 at 30°. Unlike the calculation of total hemoglobin, that of methemoglobin does not involve a correction for physically dissolved CO. The physically dissolved CO is practically the same in both blood samples, A and B, saturated with the gas under the same conditions, and hence does not affect the difference between them, due to combined CO.

Substitution of Illuminating Gas for Pure Carbon Monoxide.—The common type of illuminating gas is about one-third CO, and we find that the New York gas may be used in place of CO without significantly decreasing the accuracy of the methemoglobin estimation.

If total hemoglobin is to be estimated, however, the solubility of the gas must be determined by saturating about 15 cc. of water with the gas in another separatory funnel, and determining the gas content of the saturated water. 5 cc. of the saturated water are transferred to the blood gas apparatus and the gases are extracted without addition of reagents. The extracted gases are treated with 1 N sodium hydroxide solution to remove any CO_2 which may be present, and are then measured by reading the pressure before and after ejection from the apparatus, as in the above described CO determination. The gas content of the water is multiplied by 0.85 to estimate the amount dissolved under the same conditions in blood.

The solubility coefficient of illuminating gas is likely to be twice that of CO, and probably the results for total hemoglobin obtained with it are subject to somewhat more error than when pure CO is used. We have not found significant differences in the content determinations we have carried out, but illuminating gas made from coal varies in its composition, and whether all types of gas will yield good total hemoglobin figures one cannot predict.

Results obtained with the method above described are given in the accompanying paper by Van Slyke and Vollmund.

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STUDIES OF METHEMOGLOBIN FORMATION.

BY DONALD D. VAN ŞLYKE AND ERIK VOLLMUND.

(*From the Hospital of The Rockefeller Institute for Medical Research, New York.*)

Conant (1923) and Conant and Fieser (1925) have shown that it is characteristic of methemoglobin that by proper reducing agents it is transformed into reduced hemoglobin, with restoration of ability to combine with oxygen and carbon monoxide. The ability to be changed by reduction from a substance incapable of binding molecular O_2 or CO into reduced hemoglobin appears to be the most specific chemical characteristic of methemoglobin that we know. Determinations of methemoglobin based on it may therefore be considered to be more certain to measure that substance, and exclude other hemoglobin derivatives, than may qualitative spectroscopic examinations or quantitative estimation of loss of oxygen capacity, uncontrolled by reduction to reduced hemoglobin. The methods of Conant and Fieser ('1925), of Nicloux and Fontès (1924), and the modification of the latter in the preceding paper by Van Slyke (1925), include this control. It appears desirable to review experimentally with such methods the effects on hemoglobin of some of the substances that have been considered to transform hemoglobin into methemoglobin.

We have, accordingly, with the technique described in the preceding paper, studied the effects on hemoglobin of aniline, nitrobenzene, ferricyanide, and nitrite, which represent four different types of supposed methemoglobin formers.

Aniline.

Time Reaction of Aniline and Hemoglobin in Blood.

To 100 cc. of horse blood containing 11.3 millimols of hemoglobin per liter (by CO capacity) 4 mols of aniline were added per mol of hemoglobin (the amount of aniline calculated for 4 mols per mol of Hb is $93 \times 11.3 \times \frac{1}{1000} = 104$ mg. The amount added was 0.10 cc.) The aniline and blood were mixed and agitated in a

water bath in cylinders filled with air at 38°. A similar experiment was performed with half as much aniline. At varying periods samples were withdrawn and used for determination of total hemoglobin and methemoglobin as described by Van Slyke (1925). The blood was not hemolyzed to a significant extent. The results are indicated in Fig. 1.
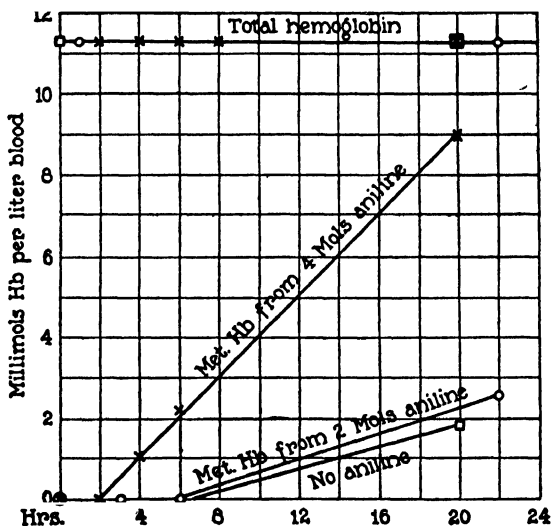
FIG. 1. Rate of formation of methemoglobin by aniline at 38°.

It is evident that the kinetics of methemoglobin formation by aniline are not simple. The outstanding qualitative facts are that:

(1) The reaction is a slow process. It is increased in rate by increasing aniline concentrations. The quantitative relationship between aniline concentration and rate of methemoglobin formation does not appear to be one of simple direct proportion, as is evidenced also by the data in Fig. 2.

(2) The reaction has a peculiar lag. No methemoglobin at all was formed by 4 mols of aniline at 38° until after 2 hours, and none by 2 mols until after 5 hours. The lag is even more marked if the reaction is carried out at room temperature, as shown by Table I.

In the experiment there recorded varying amounts of aniline were mixed with oxygenated blood and permitted to stand at room temperature. (The total hemoglobin contents in the different samples vary somewhat, apparently because of sedimentation of corpuscles

in the stock of oxalated horse blood from which the material was taken.)

The lag suggests that the methemoglobin formation is caused not by the aniline itself, but by some product into which it is changed
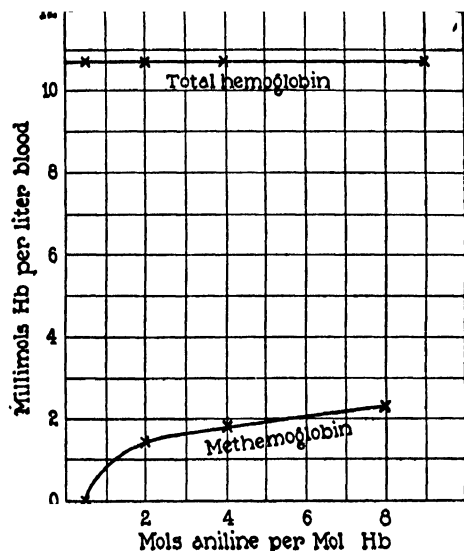
FIG. 2. Effect of variation in aniline concentration on methemoglobin formation at 25°.

TABLE I.

Lag in Formation of Methemoglobin by Aniline at 25°.

Mols analine per mol Hb.	Time of reaction.	Total Hb.	Active Hb.	Methemoglobin.
	hrs.	*mм*	*mм*	*mм*
0.5	2	9.5	9.5	0.0
2.0	2	9.3	9.3	0.0
4.0	4	9.5	9.5	0.0
8.0	4	11.3	11.3	0.0
8.0	16	8.8	6.4	2.4

by the blood. Ellinger (1920) obtained evidence that acetanilide *in vivo* is converted into acetyl-phenyl-hydroxylamine by change of the C_6H_5-$\dot{N}H$ group to C_6H_5-$\dot{N}(OH)$. It is possible that aniline is changed similarly. From the chemical structure of aniline itself

it would be difficult to imagine how it would act either as an oxidizing agent or a "hydrogen acceptor." Heubner and Rhode (1923) found that phenyl-hydroxylamine is in fact a former of methemoglobin. Lipschitz and Weber (1924) found that $C_6H_5NH_2$ and $C_6H_5NH(OH)$ have no effect on reduced hemoglobin, but form methemoglobin in the presence of oxygen. A possible explanation for the formation of methemoglobin in blood by aniline is that the latter is converted into
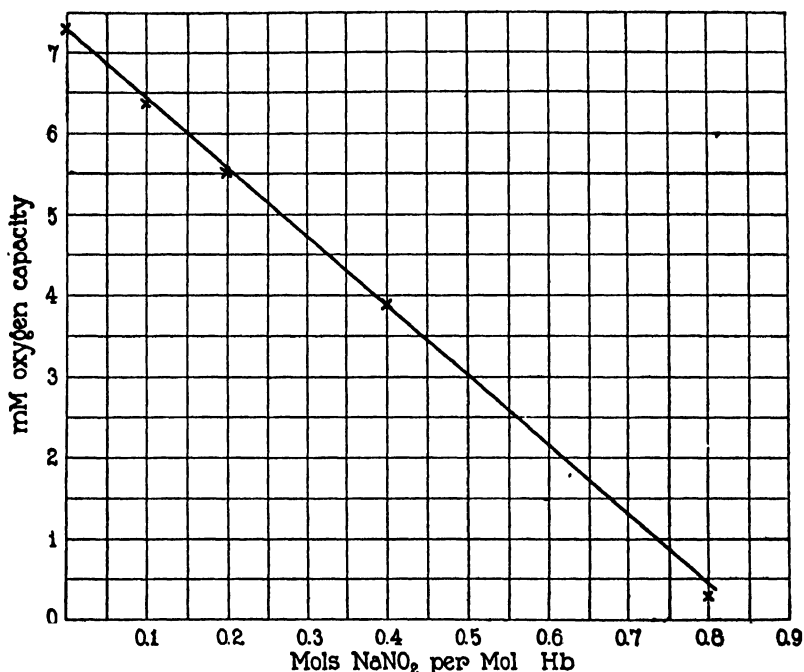
FIG. 3. Approximate formation of 1 mol of methemoglobin per 1 mol of nitrite at 25°.

phenyl-hydroxylamine, which acts as a catalyzer to accelerate the oxidation of reduced hemoglobin by molecular oxygen to methemo-globin. The scope of the present work has prevented further testing of this hypothesis.

We have attempted to produce methemoglobin formation in rabbits *in vivo* by painting the shaven abdomen with aniline to simulate the conditions under which human aniline intoxication usually happens. As in Stadie's (1921) attempts to produce methemoglobinemia in

rabbits, however, the result was only anemia. Presumably methemoglobin is formed, but does not stay in the blood. The results of an experiment, in which the painting was repeated daily for 4 days, are given in Table II. The skin at the end of the experiment was necrotic.

That the aniline penetrates the blood cells and forms methemoglobin within them *in vitro* is indicated by the following experiment.

Horse blood was mixed with 2 mols of aniline per mol of hemoglobin, and left overnight at room temperature. The cells were then centrifuged and washed several times. The saline suspension was dark brown and gave the following CO capacities:

After treatment with $Na_2S_2O_4$.................... 6.62 mM per liter.
Without   "   "   "   .... 4.48 "   "   "
Methemoglobin................................ 2.14 "   "   "

TABLE II.

Result of Painting Rabbit's Abdomen 4 Days with Aniline.

	Total Hb.	Active Hb.	Met. Hb.
	mM	*mM*	*mM*
Before painting.............................	8.2	8.2	0.0
After "	5.5	5.5	0.0

The fact that aniline *in vivo* causes a disappearance of hemoglobin (presumably after transformation to methemoglobin) instead of the appearance of methemoglobin indicates an effect of factors, presumably hemolytic, which do not act *in vitro*.

The above results demonstrate (1) that the product formed from the action of aniline on hemoglobin is genuine methemoglobin, so far as we can characterize it at present, (2) that the process of formation is a slow one, with a peculiar latent period during which no formation at all occurs, and (3) that the process can occur in the cells, which therefore appear permeable either to aniline or to whatever product of it produces the change to methemoglobin.

Nitrobenzene.

Nitrobenzene is one of the substances usually listed as methemoglobin formers. It is certain that it can reduce the oxygen-combining capacity of blood *in vivo*, but whether the inactive substance into

which the hemoglobin is changed is methemoglobin or some other derivative appears less certain. Loeb, Bock, and Fitz (1921) in two men with nitrobenzene poisoning found the oxygen capacity of the blood reduced to 6.2 and 8.9 volumes per cent respectively. The red cell count and total hemoglobin by Stadie's method were normal, corresponding to about 20 volumes per cent of oxygen capacity. The differences between total hemoglobin and oxygen capacity would indicate that over half the hemoglobin was inactivated in each case. Yet no methemoglobin could be detected by the spectroscope.

We have attempted to simulate human nitrobenzene poisoning in rabbits by painting the skin with the substance, and by administering it with a stomach tube. The effect seems quite different from that in man. Relatively much more severe treatment appears re-

TABLE III.

Effect of Nitrobenzene on Carbon Monoxide Capacity of Blood Determined with and without Hydrosulfite.

Blood.	Nitrobenzene.	Approximate mols nitrobenzene per mol Hb.	CO capacity of blood.		
			Without nitrobenzene or hydrosulfite.	With nitro-benzene without hydrosulfite.	With nitrobenzene and hydrosulfite.
cc.	*cc.*		*mM*	*mM*	*mM*
5	0.05	10	8.4	8.3	7.9
10	0.006	0.5	9.6	9.5	8.9

quired to produce intoxication in the rabbit, and the effect on the blood is to produce not inactive hemoglobin or methemoglobin, but anemia. Thus, an animal killed by 5 days painting of the abdomen showed at death the oxygen capacity reduced to 4.5 volumes per cent, but there was no evidence of methemoglobin. The spleen was filled with dark pigment.

If nitrobenzene and blood are mixed *in vitro* the mixture exhibits a peculiar behavior. The nitrobenzene produces no immediate decrease in the CO-combining capacity of the blood untreated with hydrosulfite. When the blood is treated with hydrosulfite, however, an actual decrease occurs in the CO capacity, as though the hydrosulfite, instead of changing methemoglobin back to reduced hemoglobin, had the reverse effect (see Table III). It is evident that the

hydrosulfite-CO methemoglobin method is not suitable for determination of the product formed by the action of nitrobenzene on hemoglobin.

Ferricyanide.

Potassium ferricyanide, as shown by common experience in blood oxygen determinations, and by Conant's (1923) electrometric titrations, reacts almost instantly with dissolved hemoglobin to form methemoglobin. The ferricyanide seems quite unable to penetrate the cells, however. Even after some hours standing with equimolar amounts of ferricyanide we have found blood with intact cells to show but a trace of methemoglobin formation. The presence of ferricyanide in the amounts used did not interfere with the hydrosulfite-CO method.

The following experiment demonstrates the impermeability of the cells to ferricyanide.

10 cc. of a suspension of horse erythrocytes in 0.9 per cent NaCl solution (hemoglobin content = 4.7 mM) were treated with 0.5 mol of potassium ferricyanide per mol of hemoglobin and kept 4 hours at 38°. The cells were washed several times with saline solution, and were finally suspended in saline solution and analyzed for methemoglobin.

	mM
CO capacity after hydrosulfite	3.72
" " without "	3.76
Methemoglobin	0.0

As a control, blood hemolyzed with saponin was treated with 0.5 mol of ferricyanide per mol of hemoglobin.

	mM
CO capacity after hydrosulfite	11.32
" " without "	4.53
Methemoglobin	6.79

Stadie (1921) found that relatively large amounts of ferricyanide were necessary to cause disappearance of hemoglobin from rabbit blood, and that the rate of disappearance was much slower than after nitrite injection. Presumably the impermeability of the cells to ferricyanide is responsible for the difference. It may also be responsible for the relatively slight toxicity of ferricyanide administered orally. It is furthermore the impermeability of the cells for ferricyanide that

necessitates complete laking of blood before the oxygen content can be determined by the ferricyanide method.

Nitrites.

Nitrites, like ferricyanide, quickly change active hemoglobin to a form which does not bind O_2 or CO. That nitrite hemoglobin is the same methemoglobin as that formed by the action of ferricyanide or spontaneous oxidation of partially oxygenated blood appears uncertain from the results of Hartridge (1920). The product does, nevertheless, react with hydrosulfite like methemoglobin, with regeneration of reduced hemoglobin.

10 cc. of a cell solution containing 4.3 mM of Hb by oxygen capacity measurement were treated with 10 mg. of $NaNO_2$ (3 mols per 1 mol Hb). After a few minutes the solution was divided into two parts, and the CO capacities were determined with and without hydrosulfite, as described for methemoglobin determination.

	mM
CO capacity with hydrosulfite	4.5
" " without "	0.4
Methemoglobin	4.1

Unlike ferricyanide, nitrite penetrates the red cells, as shown by the following.

A suspension of washed horse cells in saline had oxygen capacity of 8.1 mM. Two portions were treated, one with 5 mols of potassium ferricyanide per mol of hemoglobin, the other with 5 mols of sodium nitrite. After 30 minutes both suspensions were centrifugated, washed, made up to original volume, and the oxygen capacities were redetermined.

	mM
Suspension treated with nitrite, O_2 capacity =	0.89
" " " ferricyanide, O_2 " =	8.15

The rapidity of the action of nitrite is indicated by the following experiment.

A hemoglobin solution was prepared by laking 5 cc. of horse erythrocytes in 10 cc. of water. The oxygen capacity of the solution was 6.70 mM. To 10 cc. of the solution 5 mg. of $NaNO_2$ were added (1.07 mols nitrite per mol hemoglobin) and the oxygen capacities were determined at intervals. (See Table IV.)

The fact that approximately 1 mol of nitrite reacts with 1 mol of hemoglobin is shown by the following experiment.

A solution of cells containing 7.30 mM of hemoglobin, by oxygen capacity measurement, was divided into 5 cc. portions. To four such portions were added respectively 0.05, 0.1, 0.2, and 0.4 cc. portions of 1 per cent $NaNO_2$ solution, containing 0.1, 0.2, 0.4, and 0.8 mols of nitrite per mol of hemoglobin. (The $NaNO_2$ stock solution was standardized by permanganate titration.) After 30 minutes at room temperature the oxygen capacities were redetermined. The results are given in Fig. 3.

The permeability of the cells for nitrite is presumably the cause for the facts noted by Stadie (1921), that the effect of nitrite on the blood of rabbits *in vivo* occurs in a few minutes, that intravenously injected nitrite causes the formation of about equimolar amounts of methemoglobin, and that the methemoglobin remains in the cells.

TABLE IV.

Rate of Reaction of Nitrite and Hemoglobin.

Time after addition of nitrite.	Oxygen capacity.
min.	*mM*
0	6.7
5	1.5
20	0.4
40	0.4
60	0.4

SUMMARY.

Nitrobenzene acting on blood *in vitro* did not yield a product determinable as methemoglobin by the hydrosulfite-CO method. When absorbed by rabbits nitrobenzene caused anemia, without methemoglobinemia.

The actions of aniline, ferricyanide, and nitrite on hemoglobin in the presence of air yielded products which were identical with methemoglobin in that they could not bind molecular O_2 or CO until converted by reduction with hydrosulfite into reduced hemoglobin.

The action of aniline showed a latent period at the beginning, no methemogobin being formed for a time that might extend for some hours. After methemoglobin formation began it proceeded slowly, and several mols of aniline per mol of hemoglobin were required to

complete it. The behavior accords with the possibility, indicated by previous writers, that a product of aniline rather than aniline itself causes the methemoglobin formation. Aniline forms methemoglobin in the cells of unlaked blood: consequently either aniline or its product penetrates the cells.

Nitrite and ferricyanide alike react almost instantly in laked blood to form methemoglobin, and 1 mol of each forms approximately 1 mol of methemoglobin.

Nitrite and ferricyanide differ in that nitrite penetrates the cells instantly and forms methemoglobin within them. The cells, on the contrary, appear entirely impermeable to ferricyanide anion, and methemoglobin formation by ferricyanide *in vitro* occurs only after they are laked.

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THE EXCRETION OF AMMONIA AND TITRATABLE ACID IN NEPHRITIS.

By D. D. VAN SLYKE, G. C. LINDER, ALMA HILLER, L. LEITER, and J. F. McINTOSH.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

As the result of past metabolic studies, especially those of Henderson and Palmer (12) it is known that the non-volatile acids produced in excess of fixed base by human metabolism are excreted in the urine in two forms, viz., as free acids, and as ammonium salts.

Since the kidney is unable to form urine with a pH much lower than 5.0, it can excrete, in significant amounts, free acids of only the weak buffer type. In this class fall acid phosphates and the various organic acids. It appears, however, that free acid excretion may assist also in elimination of strong acids, which can react with buffer salts (e.g., $HCl + Na_2HPO_4 = H(NaHPO_4) + NaCl$), the free buffer acid being excreted in place of the strong acid. Thus Marriott and Howland (19) found that HCl ingestion increased the output of free buffer acids.

Ammonia serves to neutralize either weak or strong acids. Consequently both ingestion of HCl, (either as such, or as NH_4Cl or $CaCl_2$) and diabetic ketosis (producing weak beta-hydroxybutyric acid) cause increase in ammonia excretion. In fact both the above conditions increase both ammonia and free buffer acid output, without greatly altering the NH_3/acid ratio (Marriott and Howland (19), Fitz and Van Slyke (4)). Peculiarly, however, ingestion of acid phosphate was found by Marriott and Howland to increase only the output of titratable acid, without any effect whatever on the ammonia output.

Since the nature of the acid eliminated, and presumably other unknown factors, can influence the proportions excreted as ammonium salts and titratable free buffer acids respectively, it is not surprising

that, as shown by Henderson and Palmer, and by our data, wide fluctuations occur in the NH_3/acid ratio even in the same normal individual. To take an extreme case, when a vegetarian diet produces an alkaline urine the NH_3/acid ratio may approach infinity, because the acid factor in the denominator approaches zero, while the ammonia in the numerator, although greatly reduced, is still measurable.

Nevertheless the ammonia in a series of urines from an individual with a normal excretory mechanism ordinarily averages from 1 to 2.5 times the free acid, and this ratio is maintained over great ranges of total NH_3 + acid output, e.g., from a low normal 300 cc. of 0.1 N NH_3 + acid per 24 hours to the 4,000 or 5,000 cc. that may be excreted in diabetic acidosis. There is apparently some tendency towards a moderate increase of the ratio in the latter condition. (In the data of Fitz and Van Slyke (4) the normal urines show, as do the normal data of the present paper, a mean NH_3/acid ratio of approximately 1.5 while the mean for the cases with ketosis is 2.2. This difference in the ratio is small, however, considering the immense range of total NH_3 + acid values covered.) The eliminated acid is divided between ammonium salts and free acid with a sufficient degree of constancy to justify the definition of the usual limits of the NH_3/acid ratio in the urine of normal individuals, and the assumption of an abnormality in the acid excreting mechanism when this ratio consistently lies outside such limits. A persistent abnormally high ratio may result from bacterial formation of ammonia from urea in the bladder. A persistent abnormally low ratio is presumably a sign of damage to the ammonia forming function, particularly when the titratable acid factor of the ratio appears unaffected.

Henderson and Palmer (12, 11) determined the free acid by titrating the urine to pH 7.4, the reaction of normal blood plasma. The NH_3/acid ratio[1] averaged over a number of days in the urine of normal subjects varied from 0.7 to 2.0. Nephritic cases fell into 2 groups.

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[1] Henderson and Palmer expressed their results by the ratio acid/NH_3. We have inverted this ratio and use the expression NH_3/acid, because (1) the relative rise and fall in the ammonia appears to be the significant factor in varying nephritic conditions, the free acid excretion being relatively unaffected. With the ammonia in the numerator, therefore, the ratio rises and falls with the more

Among the cases with normal ratios there was a predominance of conditions diagnosed as degenerative nephritis. In another group, more sharply definite in regard to urinary character, the NH_3/acid ratio varied from 0.3 to 0.6, these low values being due to diminished ammonia output. On the average in this group of cases the urine volume was abnormally great, its pH unusually low, and the "total acid output" (NH_3 + acid) diminished because of deficit in the NH_3 constituent. The low urinary pH was interpreted to indicate a tendency towards nephritic acidosis, presumably caused by the reduced ammonia formation. In this group chronic glomerulonephritis was prominent.

Stillman, Van Slyke, Cullen, and Fitz (31) reported from this hospital a case of acute nephritis following pneumonia in which there was almost complete suppression of ammonia excretion, and a progressive fall in blood alkali reserve. When the latter had dropped to about half normal, the clinical condition began to improve and the ammonia output gradually increased until it reached the unusual maximum of 1500 cc. of 0.1 N ammonia per day. The daily ammonia excretion then also fell to the usual 300 or 400 cc., presumably because there was no further need for unusual acid elimination. (Only the alkali reserve data were published, the present ammonia figures are from the hospital records.)

Rabinowitch (25) has recently shown that in diabetic patients with albuminuria the NH_3/acid ratio averages lower than in normal persons or in diabetic patients without albuminuria. He concludes that diabetics with injured kidneys have a subnormal ability to form ammonia, and are probably rendered thereby unusually susceptible to acid intoxication.

Interest both in the origin of the ammonia of the urine and in its variation in nephritis has been augmented by Nash and Benedict (21) who have shown that the ammonia is probably formed by the kidneys. It had been believed that the ammonia was formed else-

significant variable. (2) The NH_3/acid ratio, as will be shown, is low when kidney function is diminished, and in its relation to renal function it appears simpler to denote ammonia-acid relationship by an expression which parallels the renal function, rather than by one which increases when the function falls.

where in the body and carried by the blood to the kidneys. The early determinations of ammonia in blood, with the exception of those of Folin and Denis (5), were compatible with this hypothesis. Nash and Benedict found that when minute precautions were taken the blood from the carotid and vena cava of dogs contained less than 0.1 mg. of ammonia nitrogen per 100 cc. The blood from the renal vein, however, contained about twice as much, a fact which strongly suggested that the kidneys are the site of the ammonia formation. Loeb, Atchley, and Benedict (18) have repeated and confirmed these observations.[2]

In this paper we present the results of routine determinations of the NH_3/acid ratio carried out on nephritic patients over a period of several years. (Many of the patients reported in this paper have been included in previous papers on other phases of nephritic metabolism (14, 15, 16, 17.) Blood ammonia determinations on a number of patients and on normal subjects have been made to determine whether the low ammonia excretions observed in the former were due to diminished formation or to retention.

An attempt has been made to correlate the variations in the NH_3/ acid ratio with the type and severity of the cases. The latter have been classified according to the plan of Volhard and Fahr (15, 35, 36) in four groups, nephrosis, acute glomerulonephritis (stage I), chronic glomerulonephritis (stages II and III), and nephrosclerosis. Each type is reported in a table by itself, and in each table the cases are arranged as far as possible in the order of increasing severity, as judged by the clinical state and renal function tests. We realize that the Volhard-Fahr types are not separated by sharp lines, and that opinions are likely to differ in classifying certain cases. We have given in the tables such data as space permits to assist the reader in forming his own judgment.

[2] Strauss (32), using the vacuum distillation method of Hahn and Kootz (9) reported the normal blood ammonia nitrogen as 0.8 to 2.0 mg. per 100 cc. and nephritic values ranging up to 6 mg. Gherardini (8) reported 0.4 to 0.7 mg. in both normal and nephritic subjects. Such high values seem explainable only by errors of technique.

The analyses of nephritic urine were carried out on 24-hour specimens which had been preserved with toluene in an ice box from the time the individual specimens were obtained. In some cases 24-hour urine specimens were examined daily. In others they were taken every second day or once each week. In the series of determinations on normal individuals the specimens were collected over periods generally of about 2 hours, and analysed immediately.

Methods.—Urinary ammonia was determined as described by Van Slyke and Cullen (33), 5 cc. of urine being mixed with 5 cc. of saturated potassium carbonate solution and aerated into standard acid.

The *titratable acidity* of the urine was determined by Folin's familiar method (6). Because the neutrality of our solid potassium oxalate could not be trusted, we have used instead of the solid substance prescribed in the original method, a saturated solution neutralized to phenolphthalein. Fifteen cubic centimeters of this solution and 0.5 cc. of 1 per cent phenolphthalein solution were added to 25 cc. of urine; the mixture was thoroughly shaken, and was titrated to a pink color (pH approximately 8.5) with 0.1 N NaOH. Henderson and Palmer (12, 11) used neutral red as indicator and pH 7.4 as end point, a procedure which has an advantage in physiological interpretation in that the results express the amount of alkali required to bring the urine to the pH of normal blood serum. For the purpose of ascertaining the NH_3/acid excretion ratio, however, we have preferred the phenolphthalein titration because its more alkaline end point represents a urinary reaction at which the ammonia excretion becomes very small. Even in urine alkaline to phenolphthalein there is, it is true, some ammonia, and in consequence a urine with a pH of 8.5 would yield an NH_3/acid ratio of infinity. When the amount of 0.1 N alkali required for 25 cc. of urine falls below 1 cc. the NH_3/acid ratio may accordingly be higher than in urine of more usual acid content excreted by the same individual. The interpretation of the physiological and clinical significance of the ratio in such urines is therefore uncertain. They are, however, rare.

No attempt has been made to prevent escape of CO_2 from the urine. We have preferred to perform the titrations under conditions that

would be a practicable part of hospital routine, and to permit such increase in the margin of variation as might result. In any but the most alkaline urines the effect could not be important.

The Blood Ammonia—Five cubic centimeters of oxalated blood were transferred, within three minutes of the time of drawing, into the test tube of the Van Slyke-Cullen apparatus used for urea estimations, in which the rubber connections were as short as possible. An equal volume of saturated potassium carbonate, from which the ammonia had been almost completely removed by aeration for one hour, was added. A flowmeter (22) was connected, and the contents of the tubes were aerated for 5 minutes with an air current flowing at the rate of 5 liters per minute. (Van Slyke and Cullen found that with this time and air current 98 per cent of the ammonia was removed.) The ammonia was caught in a tube, calibrated at 25 cc., containing 1 cc. of 0.005 N acid diluted to about 15 cc. with ammonia-free water. As indicator we used 4 drops of a saturated solution of methyl red in 70 per cent alcohol, as suggested by Barnett (2). At the end of the aeration the tubes were washed down and diluted to 25 cc. with ammonia-free water. The excess acid was titrated with 0.005 N alkali to a standard end point color, by which all the solutions had been standardized. For this end point a buffer mixture of pH 5.6, made by mixing 91 cc. $\frac{M}{5}$ sodium acetate and 9 cc. $\frac{M}{5}$ acetic acid, was used. The standard color was that of 25 cc. of this mixture to which 4 drops of indicator had been added. The titrations were made from a 2 cc. micro burette, so that the volumes delivered could be measured to within 0.005 cc. Blank determinations were performed on the reagents, and the amount of ammonia obtained from them was deducted from that found in the blood analyses. The accuracy of this method is shown in table 1.

Table 2 shows that all of the measurable ammonia of the blood is aerated in the first 5 minutes when the rate of air flow is 5 liters per minute (controlled by a flowmeter). Further aeration, up to 40 minutes, gave no more ammonia, which proves that the potassium carbonate did not react with any constituent of the blood to produce ammonia. In fact the carbonate prevents the spontaneous ammonia formation that occurs in drawn blood. This spontaneous formation,

TABLE 1.

Recovery of Ammonia from Standard Ammonium Sulfate Solutions by the Blood Ammonia Method.

NH$_3$-N present per 100 cc. of solution	Amount of solution used for analysis	NH$_3$-N found per 100 cc.	Mean value for NH$_3$-N found per 100 cc.	Recovery
mg.	*cc.*	*mg.*	*mg.*	*per cent*
0.171	5	0.173		
		0.160		
		0.160		
		0.173		
		0.187		
		0.173	0.171	100
0.085	5	0.076		
		0.070		
		0.070		
		0.063	0.07	82.4
0.060		0.05		
		0.05	0.05	83.4

TABLE 2.

Effect of Aeration Time and Delay in Analysis on Blood Ammonia Determination.

Sample number	Aeration time	Blood NH$_3$-N per 100 cc.	Average blood NH$_3$-N per 100 cc.	Remarks
	min.	*mg.*	*mg.*	
1	First 5	0.014		
		0.020	0.017	Determinations made 5 minutes after blood drawn
	Additional 5	0		
		0	0	
	Additional 10	0		
		0	0	
	Additional 20	0		
		0	0	
	5	0.328		
		0.342	0.335	Same blood 1½ hours after drawn
2	5	0.021		
		0.014	0.017	Determination made 5 minutes after blood drawn
	5	0.042	0.042	Determination made 15 minutes after blood drawn

already noted by Parnas and Heller (23, 24), makes it important to add the carbonate to the blood immediately on drawing. The ammonia value may be doubled when the blood stands 15 minutes without addition of carbonate. If the blood is analyzed after standing $1\frac{1}{2}$ hours the increase may be 17-fold.

Blood urea determinations were made according to the method of Van Slyke and Cullen (33).

The urea concentration index was calculated from the urea content of blood and urine by a modification of the original method of Austin, Stillman, and Van Slyke. The present index[3] is calculated as

$$\frac{\text{Urine urea concentration}}{\text{Blood urea concentration}} \times \sqrt{\text{cc. urine volume output per minute}} = \frac{U}{B} \sqrt{V}$$

The simple concentration ratio $\frac{U}{B}$ represents the number of times the kidney concentrates the urea in compressing it from blood concentration, B, to urine concentration, U, when the urine volume output is the average normal 1 cc. per minute (= 1440 cc. per 24 hours). When the urine volume is less than 1 cc. per minute, the concentration U will normally be greater, and vice versa. In order to correct for this volume effect we multiply the simple ratio $\frac{U}{B}$ by the square root of the volume, an empirical correction which was found to hold for ordinary urine volumes (under 2 cc. per minute) by Austin, Stillman, and Van Slyke). In order to apply the formula to subjects of different weights, we have used the *volume per kilo body weight*, $\frac{V}{W}$,

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[3] The present formula is identical with that of Austin, Stillman, and Van Slyke, but is rearranged into concentration terms for convenience in calculation and interpretation. The former equation was $K = \frac{D}{B\sqrt{VW}}$, where D is the output of urea per time unit, B, V, and W having the same significance as above. The output, D, is the product of the volume, V, and the concentration, U. Hence $D = UV$. Substituting UV for D we have $K = \frac{D}{B\sqrt{VW}} = \frac{UV}{B\sqrt{VW}} = \frac{U}{B}\sqrt{\frac{V}{W}}$. A more detailed account of the use of the concentration index will be given shortly in another paper (20).

instead of the absolute volume. In this case we have used the cc. *hourly* output as V, since for adults of average size, W is about 60, and $\dfrac{\text{cc. per hour}}{\text{body weight}}$ approximates cc. per minute.

The concentration index $\dfrac{U}{B}\sqrt{V}$, or with the weight correction $\dfrac{U}{B}\sqrt{\dfrac{V}{W}}$, varies from 35 to 80 in normal subjects. Occasionally a single determination as low as 30 may be encountered, but we have observed no normal subjects with an index repeatedly and consistently below 35. That is, the normal adult, excreting urine at the average normal rate of 1 cc. per minute, concentrates urea in the urine to at least 35 times the blood concentration, usually between 40 and 60. The numerical values of the index with the present units of volume are 6.5 times as great as the values with the units used in the Austin, Stillman, Van Slyke index, which varied in normal subjects from 4.5 to 10.5.

The phenolsulphonephthalein output was determined as described by Rowntree and Geraghty (27). The injections were made intravenously, and a fresh standard was prepared for each test.

The serum CO_2 content was determined according to the method of Van Slyke and Neill (34).

The serum pH was determined according to the method of Hastings and Sendroy (10).

To avoid reporting individually a large number of observations on patients, the daily acid and ammonia excretions have been averaged for periods of several weeks or months. Changes in diet, treatment, or in the state of renal function were taken as reasons for starting new periods.

Analysis of Results.

. *Normal Subjects.*—(Table 3 and fig. 1.) Ninety-five determinations of the ammonia-acid ratios were made on 11 normal subjects. The collection of specimens was generally made over 2-hour periods in the course of the day. No attempt was made to control the diet, fluid intake, or activity of these subjects. The volume, specific gravity, and the absolute amounts of ammonia and acid excretion varied consider-

TABLE 3.

The Ammonia-Acid Ratio of Normal Individuals.

Number	Subject	Date	Time	Volume	NH₃ excreted per hour	Acid excreted per hour	$\frac{NH_3}{A}$
				cc.	*cc. 0.1 N*	*cc. 0.1 N*	*ratio*
1	L. L.	4/ 6/25	10.38–12.12	60	15.5	11.9	1.30
			1.00– 3.00	98	14.5	13.4	1.08
			3.00– 5.00	56	16.1	13.4	1.20
		4/ 7/25	8.20–10.20	83	11.3	8.3	1.36
			10.20–12.20	68	11.7	11.1	1.05
			1.05– 3.05	90	12.9	17.9 .	0.72
			3.05– 5.05	64	13.8	15.9	0.87
		4/ 8/25	12.30– 2.30	99	21.3	16.4	1.30
			2.30– 4.00	50	15.7	17.6	0.89
			4.00– 5.00	38	14.2	17.3	0.82
		4/ 9/25	1.15– 3.00	60	14.7	19.4	0.76
		4/16/25	8.35–11.20	119	8.4	3.5	2.40
			11.20– 2.35	128	7.7	13.4	0.57
		4/17/25	8.30–11.00	109	.9.5	4.0	2.37
			11.00– 3.15	198	. 11.7	11.4	1.03
		4/23/25	7.30– 9.15	86	8.8	5.0	1.76
			9.15–11.15	73	10.2	4.9	2.08
			11.15– 1.15	77	12.6	14.1	0.90
			1.15– 3.15	49	9.9	15.6	0.63
			3.15– 5.00	56	18.4	16.8	1.10
		4/24/25	7.30– 9.00	130	9.7	6.3	1.54
			9.00–11.00	81	12.4	10.1	1.23
			11.00– 1.00	91	13.5	14.3	0.95
			1.00– 3.00	45	14.7	15.8	0.93
Average..							1.04
2	J. G.	4/16/25	10.07–12.17	286	12.4	7.8	1.59
			2.17– 4.17	360	9.0	10.9	0.83
		4/17/25	9.40–11.40	400	8.9	3.5	2.54
Average..							1.65

<center>TABLE 3—<i>Continued.</i></center>

Number	Subject	Date	Time	Volume	NH₃ excreted per hour	Acid excreted per hour	$\frac{NH_3}{A}$
				cc.	cc. 0.1 N	cc. 0.1 N	ratio
3	J. C. B.	4/ 8/25	2.30– 4.00	55	25.9	14.9	1.74
		4/ 9/25	2.00– 3.33	42	29.9	16.8	1.78
		4/16/25	11.45– 1.45	64	24.7	14.1	1.75
		4/17/25	10.30–12.00	116	18.0	7.3	2.47
			12.00– 2.10	118	20.2	12.5	1.62
		4/23/25	6.40– 9.00	143	23.0	12.5	1.84
			9.00–11.00	157	12.4	3.7	3.35
			11.00– 1.00	78	15.2	11.8	1.29
			1.00– 3.00	52	21.5	14.7	1.46
			3.00– 4.30	43	14.3	9.4	1.52
		4/24/25	7.15– 9.15	60	19.0	10.7	1.78
			9.15–11.15	73	19.6	6.7	2.93
			11.15– 3.15	165	37.5	18.3	2.05
Average..							1.97
4	J. W.	4/11/25	9.00–11.00	107	34.7	13.8	2.52
		4/16/25	8.00–10.00	435	139.0	144.0	0.96
			2.00– 4.30	72	30.7	26.6	1.15
		4/17/25	1.00– 2.30	111	34.0	35.1	0.97
Average..							1.40
5	F. C.	4/11/25	9.00–11.00	64	22.2	7.5	2.96
		4/16/25	10.30–11.05	33	26.8	13.7	1.96
			3.00– 4.00	40	26.3	12.6	2.09
		4/17/25	9.20–11.20	78	17.7	7.5	2.36
Average..							2.34
6	W. N.	4/ 6/25	11.00– 1.00	360	29.9	18.6	1.61
			1.00– 3.00	290	42.7	33.6	1.27
			3.00– 5.00	265	38.4	24.2	1.59

TABLE 3—*Continued.*

Number	Subject	Date	Time	Volume	NH₃ excreted per hour	Acid excreted per hour	$\frac{NH_3}{A}$
				cc.	*cc. 0.1 N*	*cc. 0.1 N*	*ratio*
6	W. N.	4/ 7/25	11.05– 1.05	430	16.2	7.3	2.22
			1.05– 3.05	416	39.7	28.5	1.39
		4/ 8/25	2.10– 3.45	235	29.4	21.5	1.37
			3.45– 5.00	207	17.3	10.9	1.59
		4/ 9/25	12.30– 2.40	408	18.0	18.4	0.98
		4/11/25	8.55–10.55	552	21.8	9.6	2.27
		4/16/25	8.50–11.05	242	18.8	3.9	4.82
			1.30– 3.30	216	28.8	17.7	1.63
		4/17/25	10.30–12.15	196	7.8	4.1	1.90
		4/23/25	7.00– 9.00	529	29.3	22.4	1.31
			9.00–11.03	471	18.7	8.3	2.25
			11.03– 1.45	255	10.0	8.3	1.20
			1.45– 3.20	113	16.2	15.7	1.03
			3.20– 5.10	266	16.6	12.0	1.38
		4/24/25	7.00– 9.05	515	28.2	23.2	1.21
			9.05–11.15	455	16.3	5.4	3.02
			11.15– 1.30	308	18.6	14.1	1.32
			1.30– 3.17	180	32.3	20.2	1.60
Average...							1.76
7	V. S.	4/16/25	9.00–12.00	92	16.6	16.4	1.01
			2.00– 4.00	118	28.6	36.6	0.78
		4/17/25	11.00– 3.20	176	14.2	14.7	0.97
Average...							0.92
8	W. G.	4/ 8/25	3.10– 4.40	52	18.8	13.7	1.37
		4/ 9/25	2.18– 4.42	125	11.9	11.0	1.08
Average...							1.22

TABLE 3—*Concluded*.

Number	Subject	Date	Time	Volume	NH₃ excreted per hour	Acid excreted per hour	$\frac{NH_3}{A}$
				cc.	*cc. 0.1 N*	*cc. 0.1 N*	*ratio*
9	J. A. P.	4/ 9/25	2.05– 4.05	79	23.2	22.4	1.03
		4/11/25	9.25–11.30	110	19.4	12.7	1.53
		4/16/25	10.10–11.45	76	14.9	10.9	1.37
			11.45– 2.00	100	21.8	20.6	1.06
		4/17/25	6.50– 9.03	132	14.5	4.2	3.45
			9.03–11.25	137	11.2	4.1	2.73
		4/23/25	6.55– 9.15	124	32.2	13.3	2.42
			9.15–11.15	105	15.5	6.8	2.28
			11.15– 1.25	77	17.7	21.5	0.82
			1.25– 3.30	63	25.6	30.7	0.83
		4/24/25	6.50– 9.20	130	21.0	14.0	1.50
			9.20–11.20	86	13.6	5.6	2.43
			11.20– 1.35	70	15.8	18.7	0.85
			1.35– 3.35	65	25.1	23.2	1.08
Average:							1.67
10	T.N.	4/16/25	11.30–12.30	185	73.4	53.8	1.36
			1.30– 3.30	69	19.6	19.7	1.00
		4/17/25	8.30–10.30	374	106.2	132.6	0.80
			10.30– 3.00	184	16.1	14.5	1.11
Average							1.07
11	S. S.	4/16/25	1.15– 3.15	82	12.3	16.3	0.75
		6/ 3/25	10.10–11.45	67	10.6	6.5	1.63
			11.45– 1.45	140	8.3	6.8	1.22
Average							1.20

ably. The ratios varied between the extremes 0.6 and 4.9, but the majority fell between 0.8 and 2.5. The mean individual ratios fell between 0.9 and 2.3, the majority lying between 1.0 and 2.0, similar to those of Henderson and Palmer (13). Normal individuals show for ammonia and acid excretion the wide flexibility which seems char-

acteristic for normal kidney function. For this reason, isolated esti-
mations are of little value; the variability of the function is important
as well as the value of the ratio.

Nephrosis.—(Six cases, table 4 and fig. 1.) The patients in this
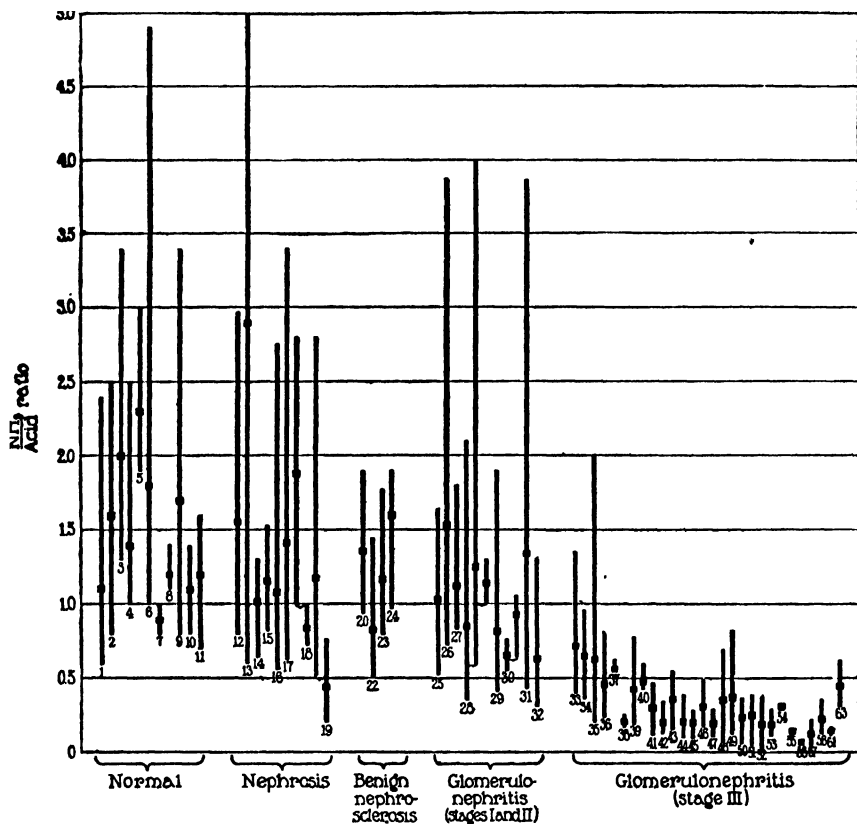
FIG. 1. The lines represent the entire range of ammonia-acid ratios for each
case. The solid blocks represent the mean ratios. Two or more lines joined
by a bracket represent different states of renal function in the same patient, as
explained in the text. The lines are numbered to correspond with the case
numbers in the tables.

group showed normal values for the ammonia-acid ratio. There was
the usual variation in range for the individual and the averages. The
ratios varied from 0.56 to 5.0, the majority falling between 0.6 and
2.8. The average of the ratios for the individual cases varied from
1.02 to 2.9, all but one lying between 1.0 and 2.0.

In B. S., 13, the ammonia was always high and the acid excretion moderate; consequently, the ratio was high. During the period from March 25 to April 18 large doses of calcium chloride were given after the method of Blum, Aubel, and Hausknecht (3); there was a rise in ammonia output which reached a maximum of 1000 cc. 0.1 N solution. This ammonia formation presumably was the protective response of the organism to prevent acidosis from the HCl absorbed from the administered $CaCl_2$ (Salvesen, Hastings, and McIntosh (29)). The calcium chloride had no effect on the edema. Between May 20 and June 14 a high protein, low fat diet was given. The protein was increased from 40 to 80 grams, and thence by successive additions to 140 grams, i.e., 4.4 grams per kilo body weight. Analysis of the diets of Sherman's tables (30) shows that the excess of acid radicles over basic was increased from 80 cc. of decinormal acid to approximately 400 cc. An increase in the output of acid and ammonia followed, but, whereas the calcium chloride produced a large increase of ammonia and a small increase of acid, the high protein diet produced a smaller output of ammonia but a larger increase of acid. A year later, during a relapse, both the titratable acidity and the ratio were still high.

In another case, M. R. (17), the administration of 5 to 10 grams of calcium chloride daily had a similar effect in increasing the ammonia-acid ratio, due to the excess excretion of ammonia over acid radicles. In this patient the pH and bicarbonate of the serum remained normal as a result of this compensatory mechanism.

In another case, G. G., 14, the lack of effect of sodium chloride is shown, where the ratio remained fairly constant, as did the absolute figures.

In nephrosis, therefore, the kidney function as regards ammonia and acid excretion is normal. This finding is in accord with the normal ability of the kidney to excrete and concentrate urea.

Table 5 shows 2 cases, B. F., and S. J., which came under observation with some of the characteristics of nephrosis, and later apparently developed renal insufficiency. In B. F., probably complicated by some other underlying condition, possibly Addison's disease, a change during the course of observation was indicated by a fall in the index of urea concentration and phthalein output. A definite drop in the ammonia-acid ratio, with a tendency to fixation, ran parallel with these findings, although the ratio was still within the lower limit of the normal. The case S. J. was under observation during two different admissions to the hospital. During the first period the ammonia-acid ratio was over 1; during the second period there were signs of definite functional impairment, and the ratio had fallen to 0.54, and finally to 0.32, at which time there was a fixation at that low value. The change in the variability of the ratio during these two periods is strikingly illustrated in fig. 1, where the first column for this patient shows a fluctuation of ratio from 0.52 to 2.8, and the second from 0.24 to 0.76.

TABLE 4.

Cases of Nephrosis.

Number	Case	Age (years)	Body weight edema-free (kg.)	Period of observation	Blood pressure	Blood urea N per liter (gm.)	Urea index $\frac{U}{V}\sqrt{\frac{U}{W/B}}$	Phtalein in 2 hours (per cent)	Plasma CO₂ content (mM.)	Plasma pH	Protein intake per day (gm.)	NH₃ excretion per day (cc. 0.1 N)	Acid excretion per day (cc. 0.1 N)	NH₃/A (ratio)	Remarks
12	F. R.	20	56	*1921* November 5–December 11		0.08	58.8	60			60–90	354	162	2.20	Acute nephrosis
				1921–1922 December 21–April 23	98/65	0.05	44.0	64			40–70	197	180	1.10	Recovered
				1922 May 5–May 23		0.06	36.8				60–70	342	139	2.46	
13	B. S.	11	33	*1922* November 5–December 27	95/70	0.07	77.3	69			60–70	313	124	2.53	CaCl₂ therapy
				1923 March 25–April 8		0.09	102.2				40	691	171	4.04	
				May 20–June 14		0.06	82.6				80–140	451	206	2.19	
		12	37	*1924* February 17–March 16	108/75	0.23*	70.4	70	22.4	7.35	45–50	266	50	5.32	Urea 30 gm. daily
				March 18–April 16		0.12*	130.0				60–70	301	116	2.60	Urea 20–10 gm. daily
		13	46	*1925* January 4–January 10	126/62	0.09	90.7	67.7			50	307	219	1.40	Recovered
14	G. G.	24	55	*1925* January 29–February 26	130/78	0.145	37.8	62.4	31.2	7.38	60	263	268	0.98	Chronic nephrosis
				February 27–March 11		0.124	35.0	65.3			75	199	178	1.12	NaCl-free diet
				March 12–March 21		0.101	34.6	68.1			75	218	243	0.90	NaCl, 10 gm. daily
				March 27–April 16		0.102	35.1	60.6			90	233	219	1.06	NaCl-free diet

15	W. J.	27	65	*1925*											
				March 13–May 2	120/72	0.05	85.2	76.8			65	264	231	1.14	
16	B. B.	23	56	*1924*											
				February 13–March 10		0.08	56.8	70	31.9	7.34	50	157	140	1.12	Urea 30–60 gm. daily
				March 11–May 28		0.31*	59.3				80	256	252	1.02	
17	M. R.	29	55	*1924*											
				December 1–December 28	106/68	0.09	39.4	75	31.6	7.46	60	142	79	1.80	
				December 29–February 12	106/66	0.16	43.5	72			100	219	384	0.57	
				February 13–March 3	110/74	0.17	40.8	60	30.5	7.41	125	494	480	1.03	
				March 12–April 6	116/78	0.07	28.2	65			60	134	116	1.16	
				April 16–May 19	120/78	0.09	31.9	60	31.3	7.37	75	645	307	2.10	CaCl$_2$ 5–10 gm. daily

* During urea therapy.

TABLE 5.

Cases First Observed with Clinical Signs of Nephrosis, and Later Developing Renal Insufficiency.

Number	Case	Age	Body weight edema-free	Period of observation	Blood pressure	Blood urea N per liter	Urea index $\frac{U}{V}\sqrt{\frac{B}{W}}$	Phthalein in 2 hours	Serum CO_2 content	pH	Protein intake per day	NH_3 excretion per day	Acid excretion per day	$\frac{NH_3}{V}$	Blood NH_3-N per 100 cc.	Remarks
		years	kg.			gm.		per cent	mM.		gm.	cc. 0.1 N	cc. 0.1 N	ratio	mg.	
18	B. F.	24	47.	*1925*												
				January 27–April 14	98/70	0.10	31.5	52	31.5	7.47	35–50	312	1661	1.88	0.02	Persistent edema
				April 14–May 19	96/70	0.21	18.4	41			50–65	170	206	0.83		
19	S. J.	33	58	*1924*												
				January 16–February 10	120/80	0.04	39.8	59	32.0	7.38	40	150	1201	1.25		Urea 30–60 gm. per day
				February 16–June 19	115/75	0.32	55.1					174	1631	1.06		
				December 16—January 15	128/80	0.17	17.6	68	29.4	7.45	40–60	101	189	0.54		
				1925												
				January 16–February 15	128/80	0.20	16.4	69	27.2	7.49	60	128	316	0.41		
				February 16–March 13	122/70	0.18	20.9	51			60	57	176	0.32		

TABLE 6.

Cases of Benign Nephrosclerosis.

Number	Case	Age (years)	Body weight edema-free (kg.)	Period of observation	Blood pressure	Blood urea N per liter (gm.)	Urea index $\frac{U}{B}\sqrt{\frac{V}{W}}$	Phthalein in 2 hours (per cent)	Serum CO_2 content (mM.)	pH	Protein intake per day (gm.)	Ammonia excretion per day (cc. 0.1 N)	Acid excretion per day (cc. 0.1 N)	$\frac{NH_3}{A}$ ratio	Remarks
20	H. Lr.	29	55	November 19–December 21 *1923*	170/120	0.15	30.4	61	28.5	7.34	45–60	282	207	1.36	Benign
21	C. T.	34	74	November *1922*	175/105	0.15	21.3				50	141	175	0.81	Benign
22	E. J.	48	54	February 8–March 5 *1922*	240/145	0.18	19.4	51	27.1		30–50	113	142	0.80	
23	M. S.	26	49	December 11–December 20 *1923*		0.11	25.2	37	24.1	7.36	55	237	183	1.30	
				December 27–January 24	196/135	0.10	25.9			7.36	55–60	124	139	0.89	
24	E. R.	43	70	April 5–June 8 *1922*	210/135	0.24	23.1	43	29.2		100	452	278	1.63	During stay in hospital apparently benign type. Died July 16 in uremia after sudden onset of severe renal insufficiency

Benign Nephrosclerosis.—(Five cases, table 6 and fig. 1.) All of these patients had only moderately diminished renal function as indicated by the urea concentration index. The NH₃/acid ratios varied from 0.5 to 1.9, with the averages from 0.83 to 1.6. These average values are within the limits of normality, but it is seen from fig. 1 that the range of variation is much less than that of normal subjects.

Glomerulonephritis, Stage I (Acute) and Stage II.—(Eight cases, table 7 and fig. 1.) In this group are included some patients who recovered completely, some with residual albuminuria and slight hematuria, and others with edema and urinary findings such as to warrant the possibility of transition into the chronic stages. However, in all these patients the history is definitely that of an acute onset, and the findings at the end of the period of observation are compatible with normal kidney function. The ratios in this group range from 0.31 to 4.0, the majority lying between 0.6 and 2.0, with averages from 0.62 to 1.53.

The first 2 cases, W. K., 25, and D. L., 26, were comparatively mild, yet their ratios increased during the course of recovery.

D. G., 28, was a severe case with marked edema (20 kilos over weight), low urea concentration index and phthalein output, increased blood urea, and some anemia. During this period the ammonia-acid ratio averaged 0.87, which is within the normal limits and, apparently, a discrepancy in view of the other findings. However, the ratio varied considerably, at times falling far below the normal limit. This variation is interesting in view of the ultimate outcome, because the patient suddenly eliminated all the edema, at which time the blood urea, urea index, and phthalein output, and hemoglobin returned to normal, while the ammonia-acid ratio increased to the usual normal value, without recurrence of the low figures. Further observations are desirable to determine the prognostic value of the variable but lowered ratio.

B. Bl., 30, was a moderately severe case which had a low normal function at the time of admission but later became normal. During the first period the ammonia-acid ratio averaged 0.65 and was decidedly fixed between 0.54 and 0.76. Later it rose to 0.93.

The output of acid and ammonia was a little low in the patient L. S., 31, during the acute stage of his illness. At the time that the edema was eliminated, the ammonia output increased and there was a temporary decrease in acidity with a rise in the ratio.

E. A., 32, showed a ratio that was persistently at the lower limit of normal, although all other functional tests were normal. Further observation is neces-

sary in order to determine the significance of such values in the face of otherwise normal functional and good clinical condition.   E. A. may exemplify a type of case in which a fixed low normal ammonia-acid ratio is the only evidence of functional disturbance in latent glomerulonephritis following apparent recovery from acute nephritis.   The urea index and phthalein output were normal.

Glomerulonephritis, Stage III (Chronic) and Malignant Nephrosclerosis.—(Thirty cases, tables 8 and 9 and fig. 1.)   This group includes patients with chronic diminution of urea concentrating power of all degrees of severity, from the early moderate forms of renal insufficiency to terminal cases with uremia and ability to concentrate only 5-fold or less.   An attempt has been made to arrange them in table 9 according to the state of renal compensation and clinical condition.   It is obvious that no sharp lines can be drawn on the basis of any functional tests; yet, on the whole, the milder forms of chronic nephritis have distinctly higher ammonia-acid ratios and urea indices than the more severe and uremic types.   As seen from table 8 and fig. 1, the first 5 cases showed moderate lowering of the urea index, phthalein output, and ammonia-acid ratio, with moderate nitrogen retention.   The average ammonia-acid ratios in the first 6 patients ranged from 0.38 to 0.88, with one higher terminal value of 1.18 in J. D., who died of septicemia. The remaining cases were of increasing severity.   In these the ammonia-acid ratio decreased to a value as low as 0.07 in one case, M. K., 56, and all were below 0.47, with the exception of S. L., 49, in whom the ratio was 0.6 during one period.   In the severe uremic cases, 58, 59, 60, 61, the ratio paradoxically rose to infinity in the terminal analyses due to an alkaline urine, which in turn may have been due to a terminal bacterial contamination of the urine.   The patients were in coma, and the resulting urinary retention, such as occurred in 61, may have led to bacterial decomposition.

In the group of chronic glomerulonephritis with diminished urea excreting function, there was a striking fixation of the ammonia-acid ratio as well as of the urea index, the only exceptions being in the terminal alkaline urines mentioned above.   It can be seen from the table that the low ratios are due to lowered ammonia excretion, never to unusually high daily acid output.

Cases V. S. and N. H., 61 and 62, are glomerulonephritis on a sclerotic basis, which would be classified by Volhard and Fahr as malignant nephrosclerosis.

TABLE 7.

Cases of Glomerulonephritis, Stage I (Acute) and Stage II.

Number	Case	Age (years)	Body weight edema-free (kg.)	Period of observation	Blood pressure	Blood urea N per liter (gm.)	Urea index $\frac{U}{B}\sqrt{\frac{B}{W}\cdot\frac{U}{A}}$	Phthalein in 2 hours (per cent)	Serum CO₂ content (mM.)	pH	Protein intake per day (gm.)	Ammonia excretion per day (cc. 0.1 N)	Acid excretion per day (cc. 0.1 N)	$\frac{NH_3}{A}$ (ratio)	Blood NH₃–N per 100 cc. (mg.)	Remarks
				1922												
25	W. K.	11	43.6	February 9–March 14	100/65	0.14	54.7	43	22.9		26–40	146	195	0.75		Initial blood pressure 165 mm.
				March 22–April 24	95/65	0.08	45.7	83	25.4		70	175	183	0.96		
				May 1–June 13		0.08	94.7	59			70	268	206	1.30		
				1924												
26	D. L.	14	28	January 3–January 24	125/70	0.10	55.0	61			30	135	150	0.90		
				February 4–March 20		0.06	47.8				40	172	93	1.85		
			32	March 23–May 4	125/65	0.06	40.7	74	27.2	7.42	40	188	134	1.40		
				1925												
27	C. C.	13	38	April 17–May 17	106/70	0.10	68.2	65			50	210	188	1.12		
				1924–1925												
28	D. G.	30	55	December 18–February 17	140/90	0.33	17.7	37	25.2	7.34	75	246	295	0.87		Marked edema
				February 18–April 7	110/75	0.09	73.8	64			100	232	181	1.28	0.01	Edema elimination
				April 8–May 12	112/76	0.09	67.6	60			75	175	153	1.14		Slight return of edema (nephrotic type)

29	A. C.	27	55	*1925* April 7–April 26	124/90	0.11	37.4	65		45	116	142	0.82	Persistent edema and hydrothorax nephrotic type
30	B. Bl.	34	49	*1925* February 8–March 10 March 11–April 21	$\frac{178}{8}-\frac{114}{70}$ 134/80	0.13 0.07	33.7 50.5	70 70	24.7 7.49	30–40 50–75	108 215	167 231	0.65 0.93	
31	L. S.	30	78	*1922* October 12–October 31 November 15–November 16 November 19–November 30 December 5–December 27	132/85 124/74	0.47 0.40* 0.19* 0.20*	12.6 17.1 36.3 28.2	44 55 62		30–40 60 70 85	136 130 226 223	236 110 88 219	0.58 1.18 2.57 1.02	Nephrotic type Edema elimination Recovered
32	E. A.	29	63	*1924* March 31–May 15	144/86	0.08	47.1	65		60	170	273	0.62	Stage II

* During urea therapy.

Cases of Glomerulonephritis, Stage

Number	Case	Age	Body weight edema-free	Period of observation	Blood pressure	Blood urea N per liter	Urea index $\frac{U}{B}\sqrt{\frac{V}{W}}$	Phthalein in 2 hours
		years	kg.	1922		gm.		per cent
33	J. D.	14	38	May 23–July 27	140/78	0.21	27.8	51
				1923				
			41	March 4–March 25	120/68	0.15	24.6	
		15	42	November 27–November 29	130/65	0.23	23.9	50
				1924				
34	F. M.	13	33	January 31–March 4	$\frac{180-180}{180-90}$	0.15	18.8	26
				March 6–March 30				
				April 1–May 8		0.20	10.0	31
				1922				
35	A. Sd.	40	54	March 5–March 29		0.26	17.5	49
				May 15–June 14		0.57	9.0	24
				1923				
36	J. O'M.	16	41	October 12–December 24	115/70	0.43	16.2	51
				December 25–March 27		0.19	20.7	46
				1922				
37	H. M.	7	18	April 8–April 19		0.40	18.4	15
				1925				
38	M. G.	12	33	May 27–May 30	128/74	0.12	31.0	62
				1922				
39	H. L.	10	29.5	May 10–June 5	160/110	0.18	31.6	41
			31	November 5–November 30	120/90	0.44	8.4	30
				1924				
40	F. P.	47	61.2	October 22–November 26	198/104	0.32	13.2	24
				1924–1925				
41	R. N.	37	58.4	December 12–January 17	150/100	0.21	20.0	46
				January 18–January 31	124/80	0.32*	24.4	60
				February 13–February 24	126/82	0.27	14.9	43
				March 4–March 16	115/80	0.19	17.6	47
				March 17–April 16	130/80	0.16	18.6	31
				1922				
42	I. C.	44	51	March 20–April 11	130/80	0.24	17.4	50
				May 18–June 4		0.31	16.2	49
				1925				
43	P. L.	28	57	January 13–January 29	164/90	0.31	14.8	55
				January 30–March 18	124/74	0.26	21.7	59

* During urea therapy.

LE 8

III, and Malignant Nephrosclerosis.

Serum CO$_2$ content	pH	Protein intake per day	Ammonia excretion per day	Acid excretion per day	$\dfrac{NH_3}{acid}$	Blood NH$_3$—N per 100 cc.	Remarks
mM.		*gm.*	*cc. 0.1 N*	*cc. 0.1 N*	*ratio*	*mg.*	
		30–50	117	157	0.75		
		70	103	162	0.64		
15–21	7.29	10	328	277	1.18		Soda bicarbonate, 27 gm. Died December 2, 1923, of septicemia
20.1	7.34	30–35	108	154	0.70		Edema elimination
		35–40	90	157	0.57		
22.3	7.38	40	130	181	0.72		Died November 1924, of pneumonia
		40–50	85	197	0.43		
		60–70	170	193	0.88		
		50	144	250	0.58		
		60–70	114	297	0.38		
		30–50	55	98	0.56		
		35	63	321	0.20		.
		50	91	209	0.43		
		45	51	169	0.30		Ascites and hydrothorax
29.0	7.40	60	112	237	0.47		Died December 6, 1924
24.0	7.44	65	115	459	0.25		
		65	94	430	0.22		Nephrotic type; urea, 30 gm. per day
25.4	7.40	100	101	359	0.28		
		60	91	208	0.44		
		75	69	249	0.28	0.01	
		60	66	297	0.22		
		70	49	226	0.22		Died May 24, 1924
26.0	7.39	60	94	345	0.27		
		75	84	246	0.34	0.09	

TABLE 8.

Number	Case	Age	Body weight edema-free	Period of observation	Blood pressure	Blood urea N per liter	Urea index $\frac{U}{B}\sqrt{\frac{V}{W}}$	Phthalein in 2 hours
		years	kg.	1923		gm.		per cent
44	S. Ly.	15	40	January 21–February 18	158/100	0.28	13.6	5
				February 20–February 22	165/102			
				March 4–March 27		0.17	13.6	7
				1924–1925				
45	J. L.	16	49	October 5–March 7	128/68	0.20	15.2	69
				May 20–May 22	142/80	0.14	34.4	57
				1925				
46	M. McC.	20	30	February 19–May 19	146/90	0.17	20.5	53
47	E. S.	29	49	April 27–May 22	200/130	0.28	9.1	16
				1921–1922				
48	M. H. A.	17	50	November 26–January 10		0.33	17.8	51
				January 12–June 13	140/80	0.36	10.2	42
				1924				
				June 30–July 17		0.40	4.1	
				1925				
		20		January 19–February 17	160/130	0.92	4.5	Trace
				February 27–March 17	155/130	0.66	3.5	
				March 22–April 21	150/130	0.84	1.5	
				April 22–May 12	130/100	1.14	1.3	
				1921				
49	S. L.	17	57	November 1–November 5	188/122	0.31	23.0	16
				November 6–November 24		0.20	9.6	20
				November 27–December 11		0.12	12.5	31
				1922				
				February 1–April 12	140/70	0.29		29
				1923				
				December 26–December 29		0.49	8.0	8
				1923				
50	M. F.	25	48	January 7–January 21	180/130	0.78	6.2	17
				January 23–February 13	155/105	0.54	5.0	20
				February 15–March 8	145/95	0.53	5.9	
				March 11–May 27	160/110	0.55	5.1	26
				1924				
51	C. A.	34	50	October 13–December 19	180/104	0.72	5.2	5
				1923				
52	E. L.	33	48	December 2–December 19	220/120	0.47	6.3	Trace
				1924				
				January 13–January 27		0.37	5.2	Trace

—*Continued.*

Serum CO₂ content	pH	Protein intake per day	Ammonia excretion per day	Acid excretion per day	$\frac{NH_3}{acid}$	Blood NH₃-N per 100 cc.	Remarks
$mM.$		$gm.$	$cc.\ 0.1\ N$	$cc.\ 0.1\ N$	$ratio$	$mg.$	
		50	36	256	0.14		
		50	60	230	0.26		CaCl₂, 12 gm. per day
21.3	7.39	50	46	191	0.24		
27.0	7.42	55	62	311	0.20		
			24	100	0.24		
24.0	7.45	40–50	61	205	0.30	0.01	
25.8	7.47	50	48	139	0.35		
23.0		30–60	132	321	0.41		
		60	90	300	0.30		
		20–40	51	122	0.42		
19.8	7.37	45	44	154	0.29		
		45	22	87	0.25		
			41	112	0.37	0.06	
			15	51	0.29		Died May 26, 1925
16.2	7.22	30	114	270	0.42		Acidosis; 60 gm. NaHCO₃
		35	55	191	0.29		
20.5	7.48	30	107	176	0.61		Edema elimination
		50	74	190	0.39		
		40	76	340	0.22		Died June, 1924
22.7	7.39	30–50	69	293	0.24		
		40–50	70	273	0.26		Edema elimination
		60–70	69	235	0.29		
		40–50	53	224	0.24		Died April 20, 1925
24.0	7.35	55	58	235	0.25		
21.9	7.34	30–40	53	176	0.30		
26.5	7.39	40–45	21	112	0.19		Died July, 1924

TABLE 8

Number	Case	Age	Body weight edema-free	Period of observation	Blood pressure	Blood urea N per liter	Urea index $\frac{U}{B}\sqrt{\frac{V}{W}}$	Phthalein in 2 hours
		years	kg.	1921		gm.		per cent
53	E. Sk.	21	56	November 18–December 11	$\frac{200-150}{140-105}$	0.60	4.5	Trace
				December 18–February 23	160/110	1.28	2.6	Trace
				1924				
54	A. S.	27	47	April 11–May 1	180/135	0.7–2.2		8
				1923				
55	J. M.	28	55	October 28–October 29	205/140	2.19		
				1923				
56	M. K.	24	44	October 5–October 6	200/105	1.58		
				1924				
57	R. S.	31	44	November 3–December 13	158/96	0.53	4.6	Trace
				December 14–January 13	170/100	0.73	4.6	Trace
				1925				
				January 14–February 17	182/112	0.82	3.8	Trace
				1923				
58	J. C.	27	60	October 25–November 13	170/117	0.42	9.1	27
				November 15–December 20		0.62	6.6	11
				1924				
				March 20–April 14	195/130	2.87	2.7	
				1925				
59	A. Bk.†	62	60	April 30	200/140	0.72		<5
60	H. F.†	38	61	April 30	180/100	2.48		<10
				1921				
61	V. S.	27	48	October 24–October 30	206/126	1.55	3.9	
				October 31–November 1				
				1924				
62	N. H.	52	53.4	October 11–October 31	212/120	0.67	5.9	14

† These cases were obtained from the Presbyterian Hospital through the courtesy of Dr. Atchley.

Autopsies were performed on J. D., M. H. A., S. L., E. L., E. Sk., A. S., J. M., and V. S., and the findings in each case confirmed the clinical diagnosis.

Acid and Ammonia Excretion in Nephritic Acidosis.—As pointed out above, in normal individuals and in nephrosis the threat of acidosis induced by administration of calcium chloride leads to a somewhat

—*Concluded.*

Serum CO₂ content	pH	Protein intake per day	Ammonia excretion per day	Acid excretion per day	$\frac{NH_3}{acid}$	Blood NH₃—N per 100 cc.	Remarks
mm.		*gm.*	*cc. 0.1 N*	*cc. 0.1 N*	*ratio*	*mg.*	
16.4		40–50	44	259	0.17		
20.5		70–90	57	248	0.23		Died March 17, 1921
23–13	7.35–7.22	30–2	21	70	0.30		Died May 30, 1924
18.0	7.30	6–0	18	127	0.14		Died October 30, 1923
15.9	7.37	30–0	9	135	0.07		Became incontinent. Died October 11, 1923
23.2	7.40	55	19	162	0.12		
		60	40	305	0.13		
21.0	7.29	60	21	184	0.11		Died May 12, 1925
		50–60	119	369	0.32		
22.7	7.31	60	620	Neutral	∞		
16.2	7.24	30–0	349	75	4.66		Died April 15, 1924
			748	Alkaline	∞	0.01	Uremia, edema; blood creatinine 4.2 mg. in 100 cc.
11.7			298	Alkaline	∞	0.02	Uremia; blood creatinine, 26.6 mg. per 100 cc. Died May 21.
18.7	7.33	50	11	82	0.13		Malignant nephrosclerosis. Uremia; blood creatinine, 24.7 mg. in 100 cc. Died November 1, 1921
			295	Alkaline	∞		
		55	44	163	0.27		Malignant nephrosclerosis

greater proportional increase in the excretion of ammonia than in that of acid, so that the ammonia-acid ratio increases. In contrast with this is the reaction of the kidney in chronic nephritis (7). Thus, in S. Ly., 44, whose ammonia-acid ratio was already low, the administration of calcium chloride led to no significant increase in the ammonia excretion or the ammonia-acid ratio. It is presumable, therefore,

TABLE 9.

Acidosis and the Ammonia-Acid Ratio in Glomerulonephritis, Stage III.

Case A. B., No. 63. Age 8. Weight 18 kg.

Date	Blood urea N per liter	Urea index	Ammonia excretion per day	Acid excretion per day	$\frac{NH_3}{4}$	Venous plasma			Alkali therapy	Remarks
						pH	CO$_2$	Cl		
	gm.		cc. 0.1 N	cc. 0.1 N	ratio		mM.	mM.		
1923										
November 27........	0.91	5.2	56	108	0.52					
November 29			40	89	0.45					
December 2.........			44	68	0.50					
December 6.........			33	59	0.56					
December 9.....: ...	1.57	1.9	21	57	0.37	7.05	8.9	89	7 gm. NaHCO$_3$	Convulsions and coma
December 10........			21	54	0.39	7.46	12.6	74	6 gm. NaHCO$_3$	
December 13........			21	52	0.40	7.20	11.6			
December 14........			18	37	0.49	7.40	15.6	91	5 gm. NaHCO$_3$	
1924										
January 1..........			26	54	0.48					
January 6..........	1.64	1.9	18	43	0.42	7.35	13.4	93		
January 10.........			18	46	0.39					
January 15.........			10	19	0.53	7.25	15.1		0.5 gm. NaHCO$_3$ daily	
January 20.........			9	26	0.35					
January 24.........			10	28	0.36	7.29	14.5		1.0 gm. NaHCO$_3$ daily	
February 2.........			6	17	0.35	7.30	16.4			
February 10........			6	18	0.33	7.17	12.7			
February 17........	3.23	0.6						64		Died February 18

that the acidosis noted in the more severe forms of nephritis (A. S., 54, and A. B., 63) was due to inability of the organism to form more than small amounts of ammonia.

The spontaneous occurrence of acidosis in a severe form is illustrated in the case of A. B., 63, a boy of 8, who had had glomerulonephritis for 3 years and who was under observation for the last 3 months of his life (table 9). There was no history of edema and the

TABLE 10.

The Ammonia Content of the Blood in Normal and Nephritic Subjects.

Subject	Urea index	Ammonia excretion per day	Acid excretion per day	$\frac{NH_3}{A}$	Blood NH₃-N per 100 cc.	Diagnosis
		cc. 0.1 N	cc. 0.1 N	ratio	mg.	
L. L.	54.0	12.9*	12.4*	1.04	0.06	Normal
W. N.	44.2	23.5*	15.6*	1.51	0.10	Normal
G. G.	34.6	218	243	0.90	0	Nephrosis
M. R.	28.2	134	116	1.16	0.01	Nephrosis
B. F.	31.5	312	166	1.88	0.02	Nephrosis
D. G.	73.8	232	181	1.28	0.01	Glomerulonephritis, Stage I
R. N.	18.6	69	249	0.28	0.01	Glomerulonephritis, Stage III
P. L.	21.7	84	246	0.34	0.09	Glomerulonephritis, Stage III
M. McC.	20.5	61	205	0.30	0.01	Glomerulonephritis, Stage III
M. H. A.	1.5	41	112	0.37	0.06	Glomerulonephritis, Stage III
A. Bk.		748	Alkaline		0.01	Glomerulonephritis, Stage III
H. F.		298	Alkaline		0.02	

* Excretion per hour.

blood pressure was not raised. There was retention of nitrogen at the time of admission and this rapidly increased. He had convulsions which were associated with extreme uncompensated acidosis; this was partially controlled with bicarbonate and no further convulsions occurred. With this persistent acidosis the excretion of ammonia and titratable acidity was small and steadily diminished. As both were equally affected the ratio did not change. Hence it appears that there is a possibility of only a moderately diminished ammonia-acid ratio

in severe forms of nephritis when the titratable acid excretion is also impaired, and acidosis develops. On the other hand, in spite of the low ammonia excretion, if the titratable acid remains at about the normal level, acidosis may be, and frequently is, absent.

Caution appears necessary in the use of calcium chloride, ammonium chloride, or any other acid forming diuretic, in the attempt to eliminate edema in patients with low ammonia-acid ratios, or with advanced glomerulonephritis. The kidneys may be unable to respond to the extra strain on the acid excreting mechanism, and acid intoxication may develop. It is desirable to follow the pH and bicarbonate of the serum closely during the use of such diuretics.

The Ammonia of the Blood.—Table 10 shows values for blood ammonia nitrogen in normal subjects and in different types of nephritis, including terminal uremia. The highest figure, 0.10 mg. per 100 cc., occurred in a normal subject, and the lowest figures, 0 to 0.01, occurred in individuals with various forms of nephritis. Similar results have been reported by Rabinowitch (26) and Russell (28), who used the Nash and Benedict technique (21). In spite of the fact that there was a markedly dminished ammonia excretion in some of the cases, the blood ammonia showed no increase. These observations indicate that no retention of ammonia occurs comparable to that of other nitrogenous constituents. The diminished ammonia output is due not to retention but to diminished formation.

SUMMARY.

In those forms of nephritis (*nephrosis and benign nephrosclerosis*) in which the urea excreting function is quantitatively unimpaired, both ammonia and titratable acid have been found to be excreted in normal amount and proportion. The usual ammonia-acid ratios in the urine in such cases exceed 1.

In acute glomerulonephritis a tendency to low ammonia-acid ratios was present in some cases, absent in others.

In those forms of nephritis with quantitatively impaired urea concentrating function (*chronic glomerulonephritis* and *some cases of acute glomerulonephritis*) there was regularly a low ammonia-acid ratio of 0.7 to 0.1 in the urine. It was due, as found by Henderson and Palmer,

to diminished ammonia output.   The titratable acidity was relatively well maintained until the final stage of complete renal insufficiency.

The drop in the ammonia-acid ratio, was not always proportional to the fall in urea excreting power nor to the severity of the clinical condition.   Cases with clinically mild chronic nephritis and urea concentration indices of 20 to 30 (instead of the usual normal above 40) may show ammonia-acid ratios as low (0.2 to 0.3) as other cases approaching uremia and with concentration indices below 5.   A possible explanation of the failure of the ammonia-acid ratio to be proportionally depressed in cases with very low urea function is that in them the ability to excrete titratable acid as well as ammonia is reduced.

The ammonia content of the blood was as low or lower in those forms of nephritis with diminished ammonia output as in normal individuals.   The fall in ammonia excretion in glomerulonephritis appears, therefore, due not to ammonia retention in the body, but to failure to form ammonia in proportions normally related to those of the acid metabolites.   The concurrence of diminished ammonia formation with diminished kidney function is compatible with the conclusion of Nash and Benedict, that ammonia is formed in the kidney.

The determination of the ammonia-acid ratio in the urine is technically simple, and consistently normal values of the ratio in repeated determinations appear to exclude the possibility of serious renal impairment.   The fall in the ratio, however, although present in all cases thus far observed with impaired urea concentrating power, does not regularly parallel the fall in the urea concentrating or excreting power.   The decrease in urea function is more closely correlated with the clinical severity of the nephritis.

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PHYSIOLOGICAL ONTOGENY.

A. CHICKEN EMBRYOS.

VII. THE CONCENTRATION OF THE ORGANIC CONSTITUENTS AND THE CALORIFIC VALUE AS FUNCTIONS OF AGE.

BY HENRY A. MURRAY, JR.

(*From the Hospital of The Rockefeller Institute for Medical Research, New York, and the Biochemical Laboratory, University of Cambridge, Cambridge, England.*)

It seems desirable that knowledge of the chemical constitution of the organism at successive stages in the life span be made more precise, so that chemical ontogeny may be correlated with functional differentiation and these data brought into line with the age-old problem of form and function. It is, moreover, of interest to make at least a preliminary analysis of growth into its principal constituent parts so that one may know the extent of growth at least in respect to water, inorganic, and organic substances.

It was with these objects in mind that the experiments included in this communication were conceived. More specifically the study consists of chemical analyses and determinations of the percentage of water, inorganic matter, glycogen, protein, and fat in chicken embryos of 5 to 19 days of incubation age. The chemical findings were checked by calorimetric tests so that the rate at which each separate substance was stored in the body and the total calorific value or potential energy of the embryo could be expressed in terms of age.

Methods.

The embryos, separately or collectively, depending upon their size, were weighed in weighing bottles, cut up into small pieces, and then dried to constant weight in an oven at 102°C. Later, alcohol was added before the embryos were cut up, with the intention of inhibiting enzyme action, but no definite evidence was found that this procedure made any difference in the result. The dried material thus obtained was used for the chemical analyses.

The nitrogen concentration of the dried substance was determined by the Kjeldahl method, and the value so obtained was multiplied by the factor 6.25 to give a rough approximation of the protein content.

The term fat is used to designate the extract obtained after washing the ground-up dried tissue with a mixture of equal parts of alcohol and ether followed by 24 hour extraction with redistilled anhydrous ether in a Soxhlet tube. The material was reground in a mortar with fine sand after the ether-extraction process had been partly completed. The flasks containing the extract were dried to constant weight over paraffin in a vacuated desiccator.

As a rough criterion of the carbohydrate content of the embryo, we analyzed for glycogen with Pflüger's method (1) following closely Cole's modification (2)

TABLE I.

Standardization of Bomb Calorimeter.

	Weight.	Total heat generated.*	Temperature rise.	Water equivalent.	Caloric value when water equivalent is taken as 324.	Error.
	gm.	*cal.*	*°C.*	*cc.*	*cal.*	*per cent*
Cane-sugar..............	1.7723	7050.0	3.032	325	3953.2	0.05
	1.7058	6783.6	2.923	321	3960.8	0.14
Hippuric acid...........	0.6742	3836.7	1.641	338†	5634.1	0.60
	0.9523	5442.9	2.342	324	5668.1	0
	0.8872	5063.8	2.177	326	5663.1	0.09
Average..................................				324		0.2 approximate.

* Total heat generated = weight of substance × caloric value (cane-sugar = 3955.2 cal.; hippuric acid = 5668.2 cal.) + weight of iron wire match × caloric value (1600 cal.).

† This result deviates to an unusual degree from the mean, and was consequently omitted in estimating the water equivalent.

except for the amounts of the solutions used. The final estimations of the glucose formed on hydrolysis were done according to Benedict's quantitative method (2). These analyses for glycogen were made with whole undried embryos. The results expressed in terms of dry weight were calculated on the basis of the values previously obtained for the water content at different ages.

The ash content was determined by careful ignition at dull redness over a Bunsen burner.

Determinations of fuel value were made in a Berthelot-Masler bomb calorimeter as modified by Kroeker.

To obtain the water equivalent for the apparatus, experiments were done with pure samples of cane-sugar and hippuric acid the calorific values of which are known (3955.2 and 5668.2 calories respectively (Table I)).
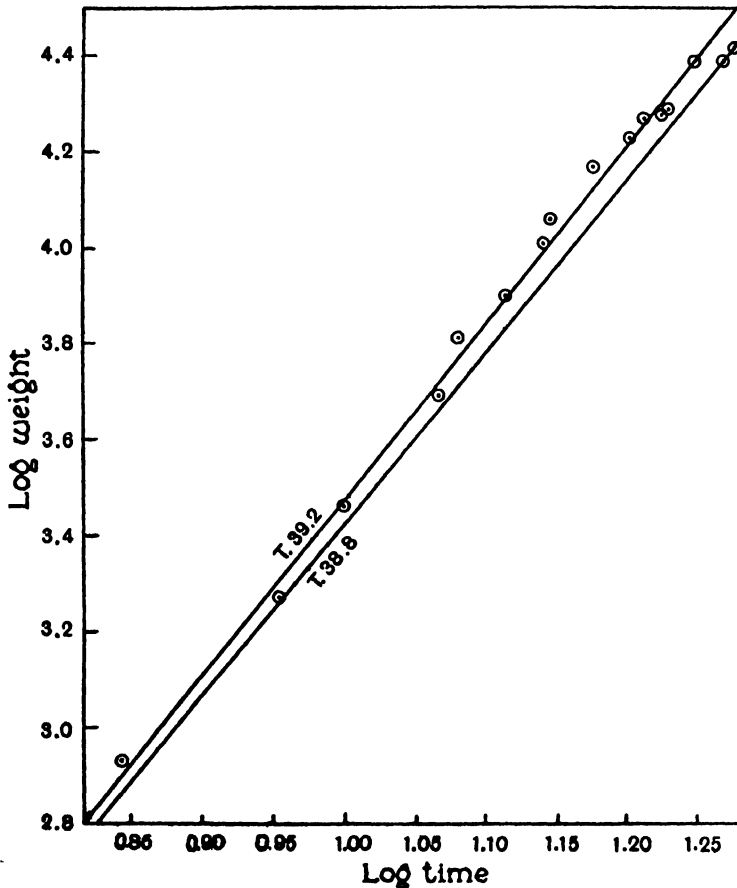
FIG. 1. The logarithm of the weight in mg. plotted against the logarithm of the time. The upper line describes the results obtained in Cambridge (T. 39.2°C.), the lower line the New York results (T. 38.8°)

The Source and Management of the Material.

The eggs used for the estimations of the nitrogen and fat content of embryos were laid during the winter (1923–24) by White Leghorn hens of 8 to 12 months of age kept on a farm in New York State. For these eggs the standards of incubation maintained were as outlined in a previous paper (3) (temperature 38.8°C.; humidity 67.5 per cent). The eggs for the glycogen and calorific value deter-

minations were likewise laid in the winter months (1924–25) by hens of a similar breed and age, but in this case on a farm in the neighborhood of Cambridge, England. The conditions of incubation for these eggs were somewhat different. Instead of a constant temperature room, an electrically heated incubator of Hearson's patent was used. The humidity varied from 60 to 70 per cent, and the temperature from 38.2–40.0°C. with averages of 67.5 per cent and 39.2°C. respectively. The eggs were rolled twice a day at room temperature. For these later (Cambridge) experiments the hens were numbered and trap-nested. It was

TABLE II.

Weights of Chicken Embryos According to Age.

(T. 39.2°C.)

1 Age.	2 No. of embryos weighed.	3 Average weight.	4 Standard error.*	5 Log weight in mg.
days		*gm.*		
7	13	0.85	0.03	2.93
9	16	1.86	0.06	3.27
10	15	2.88	0.17	3.96
11.7	4	4.85		3.69
12	10	6.39	0.34	3.81
13	6	7.94	0.48	3.90
13.8	3	10.30	0.19	4.01
14	2	11.36	0.08	4.06
15	5	14.85	0.44	4.17
15.9	4	17.00	0.68	4.23
16.3	3	18.44	0.64	4.27
16.8	2	19.16	0.91	4.28
17	7	19.38	0.48	4.29
17.7	4	24.42	0.67	4.39
18.7	6	24.77	0.52	4.39
19	7	26.59	0.55	4.92

$$* \text{ The standard error } = \frac{\text{Standard deviation}}{\sqrt{\text{No. of observations}}}.$$

possible by this means to follow the eggs from any one hen, and thus, if necessary, to diminish the extra error entailed by the greater variability of eggs chosen at random from the group.

In the second set of experiments it was noticed that the embryonic weights were somewhat higher and the date of hatching earlier than in the New York series. This difference may have been associated with the shorter period of chilling before incubation or with the slight elevation of the average incubation temperature in the former series. The embryos used at Cambridge may be compared with the much larger series collected during the period of a year and reported in a

previous paper (4) (Fig. 1; Table II). The lower of the two straight lines, corresponding to the formula log W = 3.6 log t − 0.175, was drawn to describe the points obtained when eggs were incubated at 38.8°C. In each case it was found that when the embryo reached a weight of about 25.0 gm. there was a further diminution in growth rate and the subsequent weights fell off the straight line formed when log weight was equated with log time. In the series incubated at 38.8°C., a weight of 25.0 gm. was reached on the 19th day, about 24 hours before the most advanced embryos commenced to hatch. In the later series of eggs incubated at 39.2°C. these events occurred 1 day sooner; that is, the embryos had attained an average weight of approximately 25.0 gm. on the 18th day
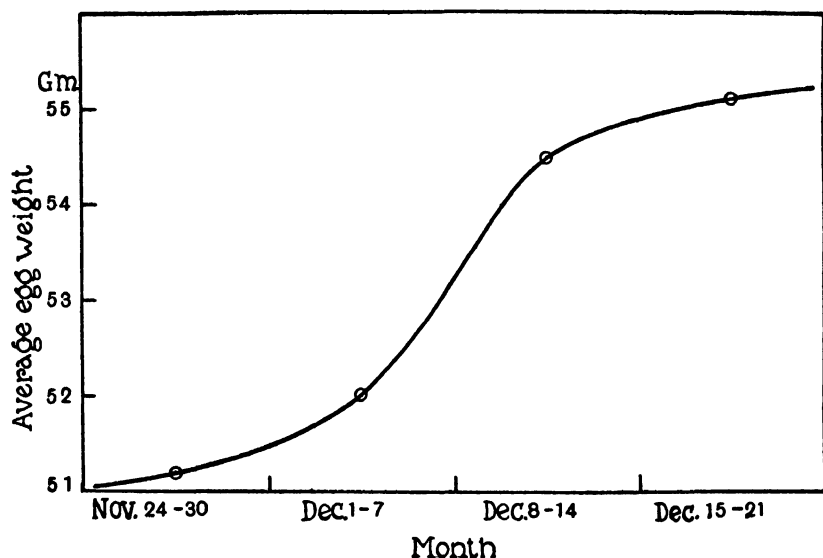
FIG. 2. To show the increase in egg weight during the early weeks after the initiation of laying.

and by the 19th day a number of the chicks had commenced to hatch, and as may be observed the point on the double logarithmic chart had fallen below the straight line. It must be pointed out that it would be a mistake to consider temperature the only factor responsible for the difference in the position and slope of the two lines since other variables were involved.

A study of correlations between the age of the hen, the chemical constitution of its egg, and the subsequent differentiation of the embryo would bear on the problem of aging. For this we have no directly relevant data. Attention is called however, to the accompanying figure (Fig. 2) which shows the change in the weekly average weight of eggs following the initiation of laying by a group of young pullet hens. It is not known in what other respects the eggs differed, but in

TABLE III.

The Dry Weight of Chicken Embryos at Successive Ages.

1	2	Solid substance			6	7	8	9	10
		by analysis.		from curve.	Wet weight embryo.	Dry weight embryo.	Daily increment dry weight.	Mid-increment dry weight.	Growth rate of dry substance.
Age.	No. of embryos.	3 Gm. per 100 gm. H$_2$O.	4 Per cent of total weight.	5 Per cent of total weight.					
days					mg.	mg.	mg.	mg.	per cent
5	136	5.62	5.32	5.32	221	11.75			
6	79	5.95	5.61	5.58	423	23.6	11.85	15.7	66.5
7	44	6.22	5.85	5.85	735	43.0	19.4	25.1	58.4
8	16	6.68	6.26	6.21	1,189	73.8	30.8	37.5	50.8
9	37	7.52	6.99	6.50	1,817	118.1	44.3	56.2	47.5
10	32	7.34	6.84	7.00	2,661	186.3	68.2	85.3	45.7
11				7.70	3,750	288.8	102.5	131.6	45.6
12	20	9.25	8.47	8.80	5,105	449.5	160.7	200.8	44.7
13	14	11.36	10.20	10.10	6,839	690.5	241.0	325.2	47.1
14	16	13.96	12.25	12.25	8,974	1,099	409.4	492	44.7
15	5	18.03	15.28	14.60	11,460	1,674	575	630	37.6
16	4	19.18	16.10	16.40	14,390	2,360	686	708	30.0
17	19	20.79	17.22	17.22	17,950	3,090	730	763	24.7
18	42	21.42	17.64	17.69	22,030	3,887	797	814	20.9
19	12	21.12	17.44	17.70	26,670	4,719	832		

The results in this table are given without their standard errors. The variability of the individuals could not be obtained as the embryos were in most cases analyzed collectively.

In Column 5 are given the values as read from the smooth curve. These will be used in the calculations involving dry weight.

In Column 6 are listed the figures for chicken embryo weights as given in a previous paper.

Column 7 = Column 5 × Column 6.

Column 8 shows the differences in the successive values given in Column 7.

In Column 9 are given the mid values between successive increments. These figures are meant to approximate the daily increment rate of dry growth.

Column 10 = $\dfrac{\text{Column 9}}{\text{Column 7}}$, or the percentage rate of dry growth.

view of the possibilities it is considered desirable to record in the future the season of the year and the age of the hens from which eggs used for experimental purposes were derived.
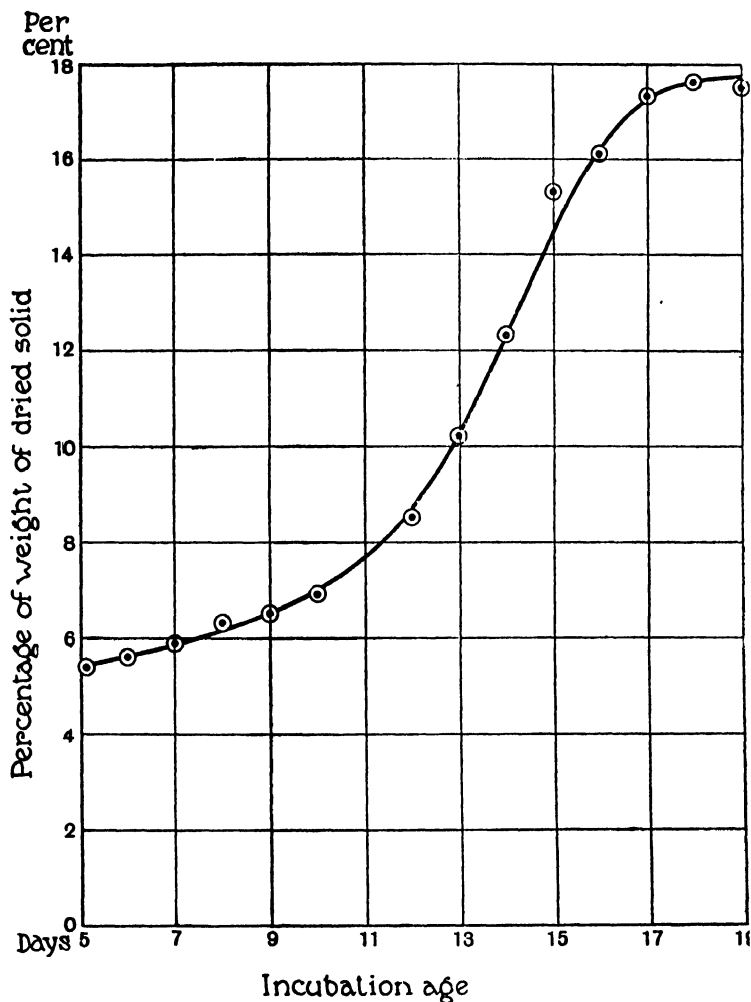
FIG. 3. The percentage by weight of dried tissue as a function of the incubation age of chicken embryos.

RESULTS.

It is apparent that the results of the chemical analyses show considerable variations. For our present purpose it seems that the data

are adequate.　Should the necessity arise for more precise measurements they may be supplemented by further observations so as to obtain more representative statistical averages for each age.

The figures for the percentage of dried solid in terms of age are fairly reliable, due to the relatively large number of determinations (Table III).　The curve (Fig. 3) shows that the proportion of solid matter to water increases with age, the most marked acceleration of

TABLE IV.

Nitrogen Content of Chicken Embryos As a Function of Age.

Age.	No. of determinations.	Average No. of embryos for each determination.	Nitrogen per 100 gm. of dry substance.	Standard error.* ±	6.25 N or protein per 100 gm. of dry substance.
days			*gm.*		*gm.*
6	8	5	11.1	0.6	69.4
7	10	5	11.2	0.2	70.0
8	4	2	11.6	0.8	72.5
9	1	1	12.2		76.2
10	3	1	11.3	0.1	70.6
11	1	1	11.4		71.2
12	3	1	10.8	0.6	67.5
13	2	1	11.3	0.5	70.6
14	2	1	11.4	0.3	71.3
15	4	1	11.8	0.4	73.8
16	1	1	10.5		65.6
17	2	1	11.1	0.2	69.4
18	2	1	11.2	0.3	70.0
19	4	1	10.0	0.2	62.5
20	2	1	9.4	0.4	58.8

$$* \text{ The standard error } = \frac{\text{Standard deviation}}{\sqrt{\text{No. of observations}}}.$$

the tendency occurring during the third quarter of the period under inspection.　This phenomenon has frequently been observed in numerous different organisms, but recorded in a more or less unsystematic fashion.　Tangl (5) pointed out that phylogeny, as well as ontogeny, showed the same tendency towards desiccation with evolution.　Aron (6) collected a large amount of data on the subject in 1913, and more recently Moulton (7) has summarized the main facts at hand and has added figures obtained by himself and coworkers from cattle and swine.

None of the available figures give an adequate account of the process during the embryonic period, especially of chicken embryos, and over no period of the life span are they sufficiently precise to be used as a basis for calculation. Although our own figures showed maximum
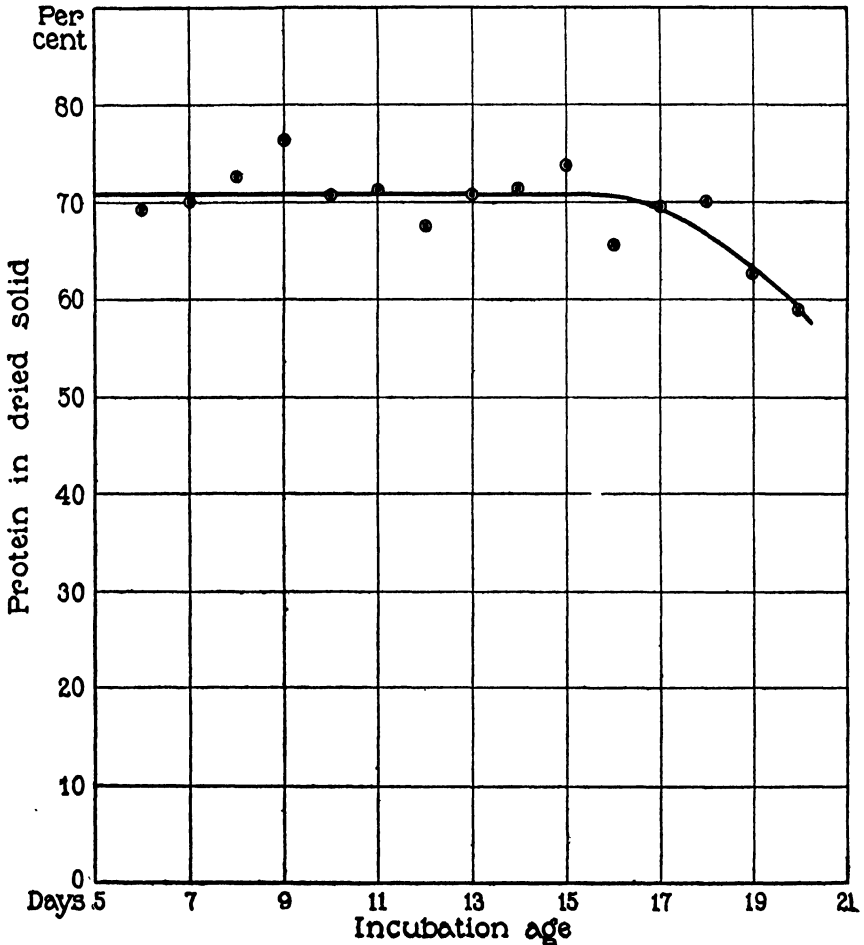
FIG. 4. The percentage of nitrogen ($\times$ 6.25) in the dried tissue of chicken embryos as a function of age.

variations of about $\pm$ 15 per cent about the mean for each age, the number of determinations and the smoothness of the curve suggest that the results have sufficient validity. Due to the importance of

water for all chemical reactions the fact that there is a gradual diminution in its percentage concentration with age is suggestive of concomitant dissipation of functional capacity. This notion has formed in the past a starting point for speculation concerning the necessary conditions of senescence.

Many other investigators have observed the fact that the nitrogen and thus presumably the protein content of the tissues increased with age, but no conclusion was possible as to whether this was the result of relative water loss or of an actual change in the proportion of the

TABLE V.

Ether Extract (Fat) Content of Chicken Embryos As a Function of Age.

Age.	No. of determinations.	Average No. of embryos for each determination.	Fat per 100 gm. of dry substance.	Standard error.*
days			*gm.*	
5	3	35	13.0	0.4
7	1	28	17.3	
9	1	24	17.5	
10	3	10	17.2	0.4
11	1	5	17.5	
13	3	7	19.0	1.5
14	1	2	17.1	
15	1	2	17.9	
16	2	2	18.7	0.3
17	14	1	. 22.6	0.5
18	7	1	- 25.4	0.7
19	15	1	28.0	0.7
20	4	1	31.5	0.7

$$\text{* The standard error } = \frac{\text{Standard deviation}}{\sqrt{\text{No. of observations}}}.$$

protein to the other solid constituents of protoplasm. Our own figures are scanty and not entirely conclusive[1] (Table IV). They may be represented graphically (Fig. 4). The curve through the average points indicates that throughout the greater part of incubation the concentration of protein in the dried tissue remains approximately 70 per cent. During the last 3 or 4 days there is a gradual decrease, which concurs in time, as will be seen later, with an increase in the

[1] The nitrogen determinations were done by Miss Alma Rosenthal and Mrs. Edith L. Wile.

fat content of the embryo. It is as if protein were displaced by fat. The figures obtained by Riddle (8) on the yolk of pigeon eggs give the reciprocal of this phenomenon.
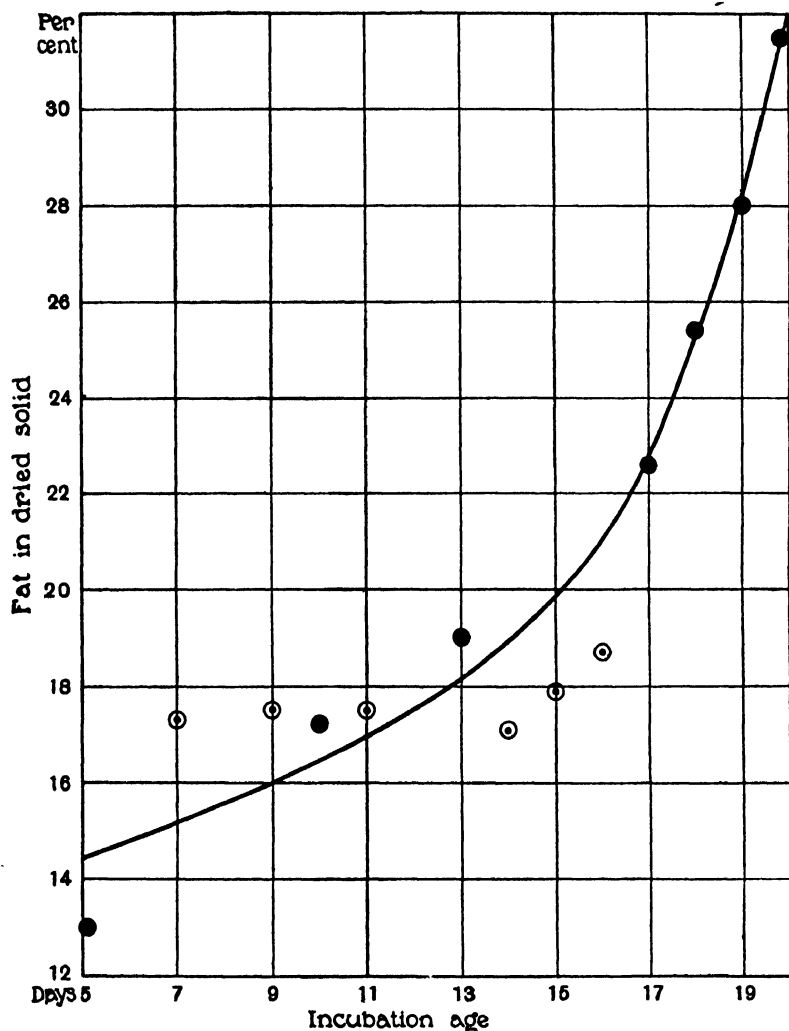
FIG. 5. The percentage of fat (ether extract) in the dried tissue of chicken embryos equated against the incubation age. The black circles represent the average of three or more determinations.

The figures for fat show deviations of similar magnitude from the mean (Table V). It is perfectly clear, however, from the graph

(Fig. 5) that there is a gradual increase in the fat content with age which is most marked during the fourth quarter of the embryonic period.   It is not at all certain what form the curve may have during the first half of incubation, but this is of minor importance.   During the time of marked positive acceleration a sufficient number of analyses were made to identify within a reasonable degree of accuracy the slope of the line.   Plimmer and Scott (9) have found that beginning with the 16–17th day of incubation there is a marked increase in the ratio of inorganic to organic (ether-soluble) phosphorus, so that it seems probable, in the opinion of these authors, that at this time glycerophosphoric acid compounds become converted into inorganic phosphate

TABLE VI.

Glycogen Content of Chicken Embryos As a Function of Age.

Age.	No. of analyses.	Average No. of embryos for each analysis.	Glycogen in whole embryo.	Standard error. ±	Dry substance in embryo.	Glycogen per 100 gm. of dry substance.
days			*per cent*		*per cent*	*gm.*
5.7	1	40	0.0085		5.5	0.155
11.7	1	8	0.0223		8.5	0.262
13.7	2	3	0.0314	0.0065	11.6	0.271
14.7	3	2	0.0561	0.0070	13.9	0.409
15.7	4	2	0.0556	0.0055	15.9	0.350
16.7	4	1	0.0610	0.0055	17.0	0.359
17.7	2	1	0.0824	0.0001˙	17.5	0.471
18.7	4	1	0.0732	0.0140	17.7	0.413
20	1	2	0.0753		17.7	0.425

for the calcification of the bones.   This suggests a considerable mobilization of fat, and is further supported by the finding that catabolism during this period is almost solely at the expense of fat.   The theoretical curves to describe the experimental points in the protein and fat charts were drawn freely with special consideration for the values representing the greatest number of observations, and also with a view of fitting the data from the other analyses.   In other words, they are *possible* curves for the special points in question and *probable* curves when the figures for protein, fat, and ash are considered together.

The analyses for glycogen (Table VI) indicate that with age there is an increase of this substance when expressed as percentage of dried

weight. At no time, however, does it exceed 0.5 per cent, which is approximately the standard error of the protein and fat determinations. For this reason glycogen need not be included in the calculations for the calorific value of the solid substance of tissues. The graph (Fig. 6) shows that glycogen increases most rapidly during the
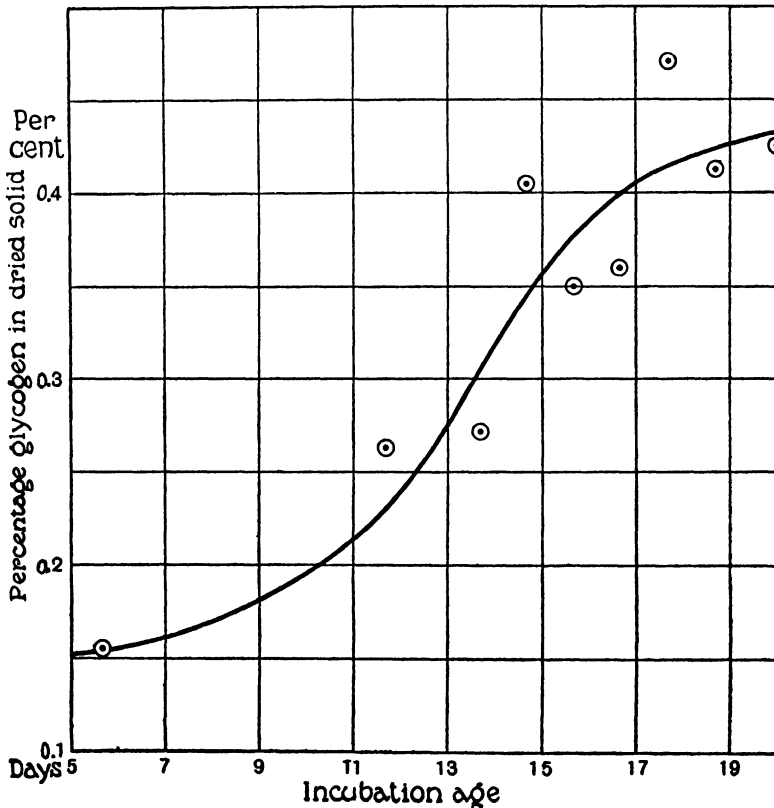
FIG. 6. The percentage of glycogen in the dried tissue of chicken embryos as a function of the incubation age.

second and third quarters of the period under discussion. Asher (10) has recently thrown some doubt on the accuracy of Pflüger's method for the estimation of this substance. By the newer method of Rona and van Eweyk (11) he obtained somewhat lower results, and suggests that substances other than glycogen are included in the total obtained by Pflüger's technique. Among those who have estimated glycogen in the organism there has been considerable disagreement.

Only one more or less systematic study of the embryonic period has been found in the literature.   Mendel and Leavenworth (12) reported for the pig embryo values increasing from 0.25 to 0.69 gm. per gm. of wet weight during the latter half of the embryonic cycle. Unfortunately the water content of the tissues was not estimated. Claude Bernard (13) discovered that glycogen was absent from the

TABLE VII.

Ash Content of Embryos in Terms of Age.

Liebermann.[*]			Author.		Theoretical figures from curve.	
1	2	3	4	5	6	7
Embryo weight.	Calculated age.	Ash per 100 gm. of dry substance.	Age.	Ash per 100 gm. of dry substance.	Age.	Ash per 100 gm. of dry substance.
mg.	*days*	*gm.*	*days*	*gm.*	*days*	*gm.*
288	5.4	15.4	10	11.0	5	14.7
1,399	8.4	13.6	10	11.1	6	14.3
2,006	9.2	14.9	12	10.5	7	13.8
2,289	9.6	12.5	13	9.8	8	13.3
8,140	13.7	10.9	13	9.6	9	12.8
12,100	15.2	7.0	13	10.8	10	12.2
16,450	16.6	7.5	14	7.9	11	13.6
16,500	16.6	8.0	14	10.2	12	10.8
18,150	17.1	7.8	15	7.4	13	9.8
18,850	17.2	7.1	16	7.5	14	8.7
19,640	17.4	7.8	19	7.8	15	7.9
20,610	17.7	7.1	19	8.3	16	7.5
21,140	17.8	5.0	19	8.3	17	7.5
22,100	18.0	9.4	20	7.7	18	7.7
640	6.6	13.6	20	8.4	19	8.0
9,358	7.5	9.5			20	8.4
26,198	19.0	11.9				

* For Liebermann's figures see No. 17 in bibliography.   Column 2 was calculated from equation $\log W = 3.6 \log t - 0.175$.

liver during the early stages of embryonic life, an observation which Gierke (14) and Lubarsch (15) later confirmed by microchemical tests on tissues.   Glycogen it seemed was to be found mostly in muscle and cartilage.   Analyses on young chicks at birth were made by Adamoff (16), who found only traces.

Omitting the carbohydrates as negligible compared to the deviations in the individual embryos and the mean errors of the other analyses,

there are at least two ways of checking our results for protein and fat. The sum of the percentage concentrations of the two latter constituents subtracted from 100 per cent should be equal to the concentration of ash; and if the proper values for the calorific value of protein and
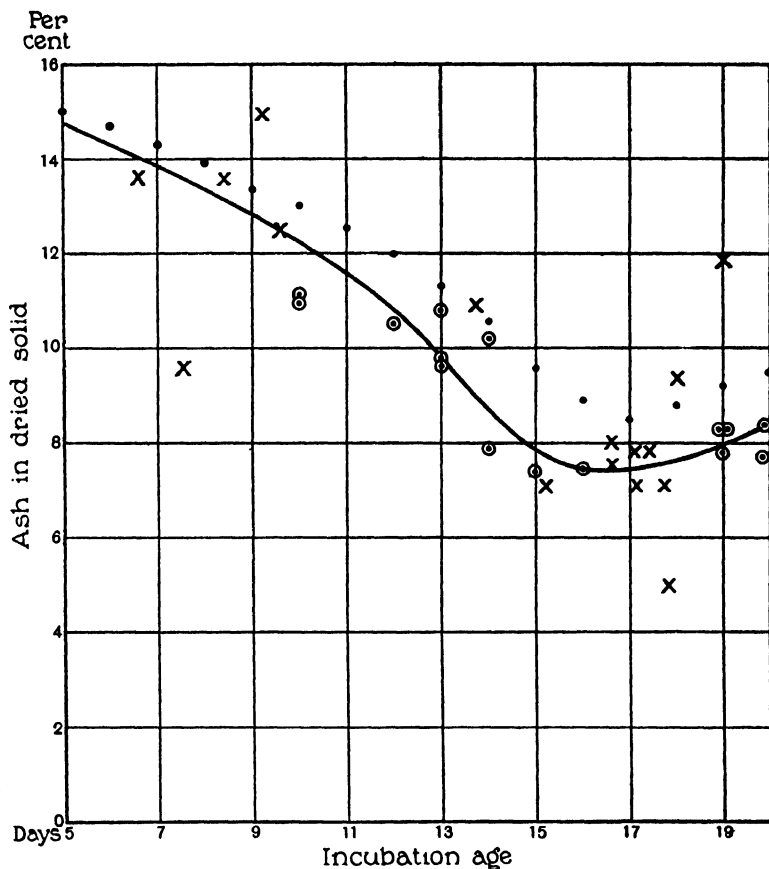
FIG. 7. The percentage of ash in the dried tissue of chicken embryos as a function of the incubation age. ╳ =Liebermann's figures; ⊙ =author's results; ● = 100 − (protein +fat).

fat were selected the products of these constants and the results derived by the chemical determinations should agree with the bomb calorimetric findings.

The figures for the ash content of chicken embryos reported by Liebermann (17) were found to conform more or less closely to our own

TABLE VIII.

Calorimetric Determinations of the Fuel Value of the Dry Substance from Chicken Embryos.

Age.	Specimen No.	No. of embryos.	Calories per gm. of dry substance.	Average for each specimen.	Average for each age.
days				*cal. per gm.*	*cal. per gm.*
7	1	17	5.202	5.202	5.202
9	2	21	5.161		
			5.151	5.156	5.156
10	3	15	5.265		
			5.204	5.235	5.235
12	4	10	5.273		
			5.279	5.276	5.276
13	5	6	5.392	5.392	5.392
14	6	1	5.510	5.510	
	7	1	5.371	5.371	
	8	1	5.535	3.535	5.472
15	9	1	5.695		
			5.638	5.666	
	10	2	5.848		
			5.814	5.831	5.748
16	11	1	5.844		
			5.845	5.845	5.845
17	12	1	5.685		
			5.733		
			5.765	5.728	
	13	1	6.077	6.077	
	14	1	6.030		
			6.162	6.096	5.967
19	15	1	5.977		
			5.978	5.978	
	16	1	5.894		
			5.981	5.938	
	17	1	6.260		
			6.221	6.241	
	18	1	6.042		
			6.102	6.072	6.057
20	19	1	6.289		
			6.219		
			6.220	6.242	
	20		.6.117	6.109	
			6.100		
	21		6.161		
			6.021	6.091	6.148

(Table VII) and were therefore used to define the curve (Fig. 7) relating inorganic matter with age.   It may be seen that the ash content of the dried substance is greatest during the early stages of life.   Later, as the time of hatching approaches, the figures suggest a slight rise which may be due to the precipitation of bone-forming salts at this period.   The small points on the chart are the values obtained when the combined figures for the percentage concentration of organic matter (protein and fat) as read from the two curves (Figs. 4 and 5)
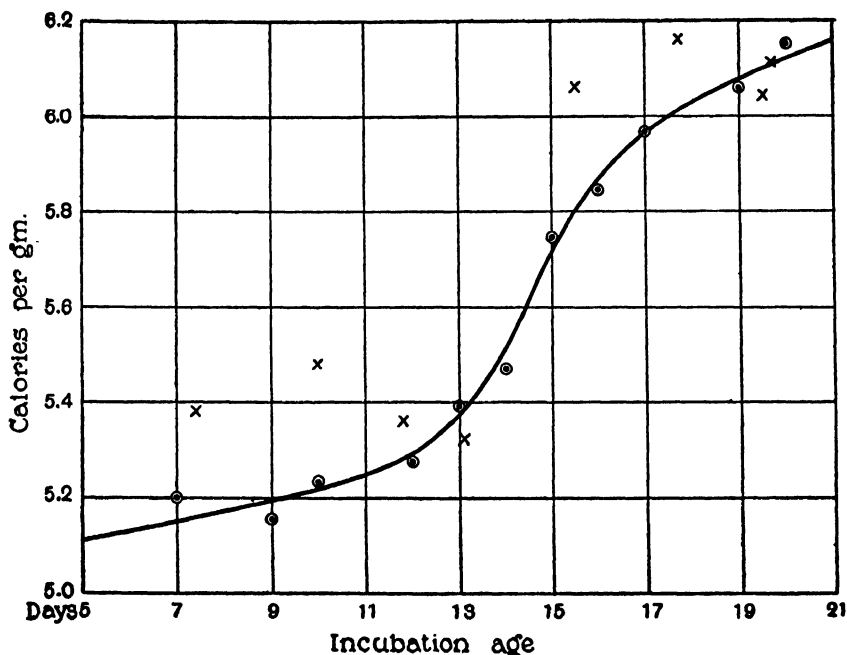
FIG. 8. The calorific value of a gm. of dried tissue as a function of the incubation age of chicken embryos.   X = Tangl's results; ⊙ = author's results.

are subtracted from 100 per cent.   (Column 5, Table XI.)   According to the curve as drawn there is about 1 per cent of the dried weight to be accounted for by carbohydrate.   This calculation, of course, is extremely crude, but the closeness of the fit suggests that 6.25 × nitrogen is a fair approximation for the protein concentration, and that a short ether-alcohol followed by a prolonged anhydrous ether extraction yields a mixture which may be considered to be mostly fat.

The results of the estimations with the bomb calorimeter (Table

VIII), together with Tangl's results (Table IX) show (Fig. 8) that there is an increase with age in the fuel value of the dried substance of the embryo. This is evidently due in part to the decrease of the inorganic and increase of the organic fractions of the solid material and in part due to the increasing proportion of fat as compared with

TABLE IX.

Calorific Value of Embryos in Terms of Age.
(After Tangl.)

1 Wet weight of embryo.	2 Calculated age.	3 Dry weight of embryo.	4 Calorific value of embryo.	5 Calories per gm. o dry substance.
gm.	*days*	*gm.*		
0.88	7.4	0.05	0.269	ʹ 5.38
2.61	10.0	0.19	1.04	5.48
4.71	11.8	0.41	2.20	5.36
6.90	13.1	0.71	3.78	5.32
13.0	15.5	2.57	15.58	6.06
20.8	17.7	3.88	23.89	6.16
24.0	18.4	4.33	27.34	6.32
29.6	19.5	6.07	36.68	6.04
30.2	19.7	5.18	31.63	6.11

TABLE X.

Calorific Value of Unincubated Eggs.

Egg No.	Test No.	Calories per gm. of dry substance.	Average for each egg.
1	1	6922	
	2	6945	6933
2	3	7084	
	4	7083	7084
3	5	6800	6800
Average..			6939

protein. With age the fuel value of the embryo approaches that of its environment; *i.e.*, yolk + albumin (Table X).

In calculating the heat developed by the oxidation of protein the nitrogen content has been multiplied by 6.25 to obtain the protein content and the result multiplied by 5.7, which is the calorific value of egg albumin and is frequently taken as a representative figure for

tissue proteins. The ether extract content multiplied by 9.3 was used as a measure of the calories from the fat fraction of the oxidized organic material (Table XI). These results may be examined graphically (Fig. 9). The circles represent the calorific value per gm. of organic material calculated on the basis described above, the curve shows the figures empirically determined (*cf.* Fig. 8). It may be seen that there
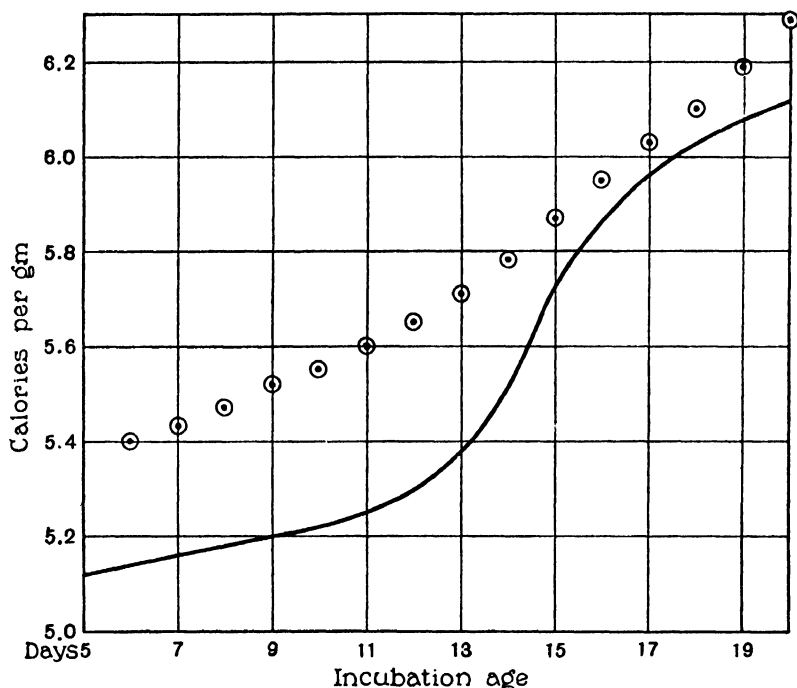
FIG. 9. The calorific value of a gm. of dried tissue as experimentally determined (————) (*cf.* Fig. 8) and as calculated from the nitrogen and fat estimations (⊙).

is a rather wide divergence in the results obtained by the two methods, more marked in the beginning than at the end of the incubation period.

The variability in the results of the actual analytical work may be judged from the figures for the standard errors given in the tables. These are entirely insufficient to account for the discrepancy. It is suggested that either or both of the two constants used in our calculations of fuel value from the protein and ether extract figures, *i.e.*

5.7 and 9.3 respectively, are too high for embryonic constituents during the early days of incubation.   For instance, if one used 5.4 instead of 5.7 for the calorific value of 1 gm. of protein, the figures would be found to agree with the calorimetric determinations.   Moreover, it

TABLE XI.

Figures for the Concentration of Inorganic Matter and the Calorific Value of Chicken Embryos in Terms of Age As Calculated from Nitrogen and Fat Estimations.

1	2	3	4	5	6	7	8
Age.	Protein.	Fat.	Fat + protein (organic matter).	Inorganic matter.	Calories per gm. of dry substance from protein.	Calories per gm. of dry substance from fat.	Calories per gm. of dry substance.
days	per cent	per cent	per cent	per cent			
5	70.5	14.5	85.0	15.0	4.02	1.35	5.37
6	70.5	14.8	85.3	14.7	4.02	1.38	5.40
7	70.5	15.2	85.7	14.3	4.02	1.41	5.43
8	70.5	15.6	86.1	13.9	4.02	1.45	5.47
9	70.5	16.1	86.6	13.4	4.02	1.50	5.52
10	70.5	16.5	87.0	13.0	4.02	1.53	5.55
11	70.5	17.0	87.5	12.5	4.02	1.58	5.60
12	70.5	17.5	88.0	12.0	4.02	1.63	5.65
13	70.5	18.2	88.7	11.3	4.02	1.69	5.71
14	70.5	18.9	89.4	10.6	4.02	1.76	5.78
15	70.5	19.9	90.4	9.6	4.02	1.85	5.87
16	70.0	21.1	91.1	8.9	3.99	1.96	5.95
17	68.8	22.7	91.5	8.5	3.92	2.11	6.03
18	66.0	25.2	91.2	8.8	3.76	2.34	6.10
19	62.5	28.3	90.8	9.2	3.56	2.63	6.19
20	59.0	31.5	90.5	9.5	3.36	2.93	6.29

Columns 2 and 3 give the values as read from the curves (figures respectively).
Column 4 = Column 2 + Column 3.
Column 5 = 100 − Column 4.
Column 6 = 5.7 × Column 2.
Column 7 = 9.3 × Column 3.
Column 8 = Column 6 + Column 7.

is clear that the difference between theoretically calculated and experimentally determined results is greater.in the early days of embryonic life.   The error involves the use of constants based on the constituents of adult tissue rather than embryonic tissue.   This, if true, would lead to an interesting fact, namely that just as the fuel value per gm.

of organic matter increased with age due to the increased proportion of fat, so also did the fuel value of either or both the protein and fat fractions rise, due to the increasing proportion, within each group, of substances with relatively high calorific values.

The figures for the fuel value may also be used for a further analysis
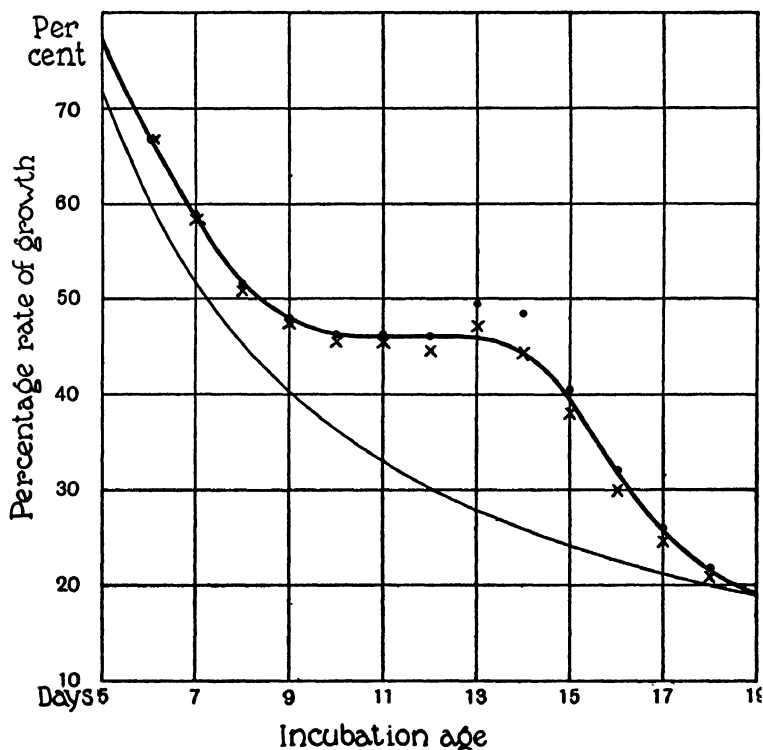
FIG. 10. The percentage rate of growth of dry solid (×) and the percentage rate of growth of calorific value (•), both described by the thick line, compared with the percentage rate of growth of the whole embryo (————) as equated against the incubation age.

of the growth problem. Not only total growth, but independently the rate of growth of the solid substances, of organic matter, or of potential energy may be studied.

In development there are two processes of major importance occurring simultaneously, (1) the integration and (2) the rearrangement of matter. The second to a certain extent may be said to counteract

the first. If there were no changes in chemical form with age, if for instance the solids "bound" as much water during the latter part of embryonic life as they do at the beginning, the embryo at birth

TABLE XII.

Growth Rate of the Calorific Value of Chicken Embryos at Successive Ages.

1	2	3	4	5	6	7
Age.	Dry weight of embryo.	Calories per gm. of dry substance.	Calorific value of embryo.	Daily increment.	Mid-increment.	Rate of growth of calorific value.
days	mg.		cal.	cal.	cal.	per cent
5	11.75	5.120	0.0602			
6	23.6	5.135	0.1212	0.0610	0.0807	66.6
7	43.0	5.155	0.2217	0.1005	0.1305	58.8
8	73.8	5.180	0.3823	0.1606	0.1962	51.4
9	118.1	5.200	0.6141	0.2318	0.2951	48.0
10	186.3	5.220	0.9725	0.3584	0.4510	46.4
11	288.8	5.250	1.5162	0.5437	0.7026	46 3
12	449.5	5.290	2.3778	0.8616	1.0976	46.2
13	690.5	5.375	3.7114	1.3336	1.8388	49.5
14	1099	5.510	6.0555	2.3441	2.9361	48.5
15	1674	5.725	9.5836	3.5281	3.8927	40.6
16	2360	5.865	13.841	4.2574	4.4162	31.9
17	3090	5.960	18.416	4.575	4.789	26.0
18	3887	6.025	23.419	5.003	5.137	21.9
19	4719	6.080	28.691	5.272		

The method of calculation used in this table is similar to that employed in estimating the percentage growth rate of dry substance (Table III).

Column 2 is the same as Column 7 (Table III).

Column 3 gives the values as obtained graphically from the smooth curve for the calorific value of dried embryonic tissue (Fig. 8).

Column 4 = Column 2 × Column 3.

Column 5 gives the differences between the values listed in Column 4.

Column 6 gives values which are midway between the increments listed in Column 5, and which tend, therefore, to approximate the instantaneous rate of growth of calorific value for the indicated age.

Column 7 = $\dfrac{\text{Column 6}}{\text{Column 4}}$, or the percentage rate of calorific growth.

would weigh approximately three times as much as it does. The percentage rate of growth for a dry substance and for the calorific value during the embryonic period may be graphically represented (Fig. 10). The method by which these figures were obtained may be understood

by examining the tables (Tables III and XII). The daily increments of energy for each age are given. As an approximation of the instantaneous rate of energy storage per gm. of embryo the mid values between successive increment values have been divided by the weight of the embryo at that age as obtained by the formula $\log W = 3.6 \log t - 0.175$. The graph shows that, as compared to the whole wet embryo there is during the mid period a temporary cessation of the decline of the growth rate. In other words, the negative acceleration for the growth rate of potential energy is greatest at the beginning and at the end of incubation. The maintenance of a constant rate during the middle of incubation may be accounted for by the great relative increase of solid substance at this period. The values for the rate of growth of the total dry weight follow those for potential energy quite closely, so that the same curve may be drawn to describe them both.

DISCUSSION.

(a)

The organism may be viewed as a locus for the absorption, storage, and elimination of chemical energy. Substances of fuel value which are absorbed may be stored without change of form, may become synthesized and stored as complex constituents of protoplasm, or may be immediately utilized as sources of energy. It would appear that the energy liberated during life is mostly at the expense of the more differentiated chemical elements of protoplasm, glycogen, protein, and fat, and that only rarely are the simpler substances directly burned in the form absorbed; that is, without undergoing a preliminary synthesis.

In view of the uncertainty about such matters the amount of anabolism cannot be estimated. The terms used in the present analysis, however, are subject to direct measurement. It is clear that:

A (absorbed energy) $= S$ (stored energy) $+ E$ (eliminated energy)

When the amounts absorbed and eliminated are equal the organism may be said to be in energy equilibrium and there will be no storage; *i.e.*, the absorption/elimination ratio or more briefly the A/E ratio, will be unity.

In this study we have measured the calorific value or potential energy per gm. of substance at each age during embryonic life. The increments from day to day give an approximate measure of the rate of storage (S).

Since $(A) = (S) + (E)$, we need only measure the daily increment of energy (ΔS) and the amount of catabolism during the same period (ΔE) to know the amount of absorption (ΔA) per day. This matter will be discussed further at a later date when analyses for the respiratory exchange have been completed.

(b)

Before undertaking these particular experiments we were impressed by what seemed to be a natural scale or gradient, as judged by various criteria, of the chief groups of substances under consideration, namely salt, carbohydrate, protein, and fat,— an order which by similar standards might likewise and with more confidence be made to apply to the substances within each group. It was thought, moreover, that since these organic substances exist in nature as constituents of living matter, progressive changes in molecular complexity would involve greater heterogeneity and thus structural and functional differentiation of the organism. Thus, the elaboration of organic substances and biological evolution might be considered as mutually dependent.

Variations and mutations may be conceived of primarily as new physicochemical states which supply the necessary conditions for new functions; for instance, more complex syntheses; and thus, with time, chemical substances of greater structural and functional complexity would come into being and these in a measure would determine histological and physiological potentialities. It seems, moreover, that with phylogenetic differentiation organisms lose the power to perform some of the simpler syntheses, for which they become dependent upon lower forms; but on the other hand they attain to more complicated chemical activity.   Just as one finds in general that along any direct line of descent, as shown for instance by the paleontological record, there has been a gradual increase in the ratio brain mass/muscle mass, so also would change, it is postulated, the ratio fat/carbohydrate. Due to the apparent uniformity of the sequences observed in similar chemical reactions and more generally in all forms of evolution, the

same general tendencies in all direct ancestral progression would be evident whether one assumed monophyletic or pluriphyletic development. Finally with ontogeny, despite one's realization of the limitations of the present modified recapitulation theories, one would expect substances of increasing complexity to be absorbed to form the more differentiated structures, or conversely that the organism, must needs become specially differentiated to utilize for its economy the more complex substances in its environment.

On this basis a tentative prediction was considered, that the following ratios would be found to decrease with age during ontogeny: water/ solid; inorganic/organic; carbohydrate/protein; protein/fat. The results reported in the present communication confirm this hypothesis in all respects except one. The carbohydrate, in so far as its concentration may be judged by the amount of glycogen in the tissues, falls out of line. It was found to follow in point of time rather than to precede the protein peak. There may be an error in using glycogen, which is present in only very low concentrations, as index of the total carbohydrate content of the embryo, but, for the present, we must call attention to this exception and let the matter stand. The facts show that the salts are found in greatest abundance at the inception of development, protein during the mid period, and fat at the very end. There are other instances in which these substances may be said to form a similar sequence.

This order holds when carbohydrates, protein, and fat are compared in respect to [1] calorific value and [2] the respiratory quotient resulting from their combustion. It is generally held that the earth has been cooling rhythmically during its history. If so, ever since the first appearance in organic evolution of a mechanism for maintaining a stable internal temperature, there would seem to be an ever increasing need for food substances of greater fuel value. Krogh and Krogh (18) have found that [3] the ratios of carbohydrate/protein and protein/fat in the average diet decrease in general as one proceeds from northern climates towards the equator. The values obtained by Bohr and Hasselbalch (19) for [4] the respiratory quotient during the chick's embryological development suggest a gradual fall with age. Needham (20) has collected evidence to show that glucose is metabolized in the beginning of incubation and it is certain, as many workers

have shown, that fat is burned almost exclusively during the latter stages. As Bohr and Hasselbalch made no correction for the CO_2 absorbed by the yolk and albumin, and therefore retained within the egg, it is probable that their CO_2 values are too low. This correction would affect principally the early part of incubation, since at this time the outside substance (*i.e.* the yolk and albumin) are very great relative to the mass of the embryo. As a result of this omission, probably Bohr and Hasselbalch's figures for the respiratory quotient are too low especially during the initial stages of embryonic life, and the fall with age is actually more pronounced than it is represented. This is another instance of the gradient.

Although there is no general agreement in the matter it is usually maintained that plant preceded animal life in geologic time. The simplest syntheses deriving their energy from the sun seem to involve [5] the formation of the lower members of the carbohydrate series. Certain it is that [6] the carbohydrates are important structural elements and are found most abundantly in plants, whereas fat and protein are more plentiful in animal tissue.

The order of ranking the substances also holds in respect to [7] the site in the gastrointestinal tract of the initial hydrolysis by digestive enzymes and [8] the location, ease, and rapidity of absorption.

Finally a very rough sort of analogy may be called to mind. The experiments of Lyon (21) and Herlant (22) on sea urchin eggs have shown that for a short time after fertilization the interdivisional period is marked by progressive changes in the susceptibility of the cells to various kinds of toxic agents. It was found that in the early stages immediately after cleavage [9] the cells were more affected by water-soluble substances, salts, KCN, acids, and alkalies, and in the later stages preceding mitotic division, by lipoid-soluble agents, chloral, acetone, chloroform, and ether. These findings were attributed to changes in permeability and it was suggested that during the development of each individual cell the constitution of the membrane changed so that at first it was more freely permeable to water-soluble, and later to fat-soluble substances, or in other words the concentration of lipoids on the surface increased with the age of the cell. This cycle for each individual cell seems to have its counterpart in the embryological cycle.

The mere enumeration of these analogies is all that we have to say for the present on the subject of chemical evolution in relation to ontogeny.

SUMMARY.

1. The water, ash, glycogen, nitrogen, and ether extract content of the tissues of chicken embryos were determined between the 5th and the 19th days of incubation.

2. It was found that the concentration of solid substance changed from approximately 5 to 17 per cent during this interval. The chief change in the organic substances involved a relative decrease in the nitrogen as compared with the fat.

3. Bomb calorimetric estimations confirmed these findings. It was shown that the calorific value of the dried substance increased with age.

4. The rate of growth of the total solids, and of the potential energy of the tissues was estimated.

5 Various theoretical considerations were brought forward dealing chiefly with the order in which the chief organic substances, carbohydrate, protein, and fat could be ranged when judged by various criteria.

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A STUDY OF ANAEROBIC BACTERIA.

BY J. HOWARD BROWN.

(*From the Department of Animal Pathology, The Rockefeller Institute for Medical Research, Princeton, N. J.*)

The very excellent work done in the study of anaerobic bacteria isolated from wounds during the World War has indicated certain directions along which the sporulating anaerobes in general may be profitably studied. Some very useful media have been introduced, notably the cooked meat or cooked brain media. Better and simpler methods of securing anaerobic conditions have been devised, such as various developments of the anaerobe jar of McIntosh and Fildes (1916) and the use of vaseline on the surface of media in test tubes. The contributions of Wolf and Harris (1917, 1918, 1919), Bushnell (1922), Kendall, Day, and Walker (1922), and DeBord (1923) demonstrated the usefulness of biochemical methods for the study of the anaerobes and the impossibility of understanding their activities without resort to such methods. The cultural and systematic studies of Weinberg and Sequin (1918), Reports of the British Medical Research Committee (1917, 1919), Robertson (1916), McIntosh (1917), Henry (1917), Adamson (1919), Heller (1920, 1921, 1922), and Hall (1922) have done much to untangle the descriptive literature of the past and to establish the identity of certain species and certain natural groups of anaerobes. With this background of methods and the beginning of a natural classification it was thought worth while to study a fairly large group of anaerobes taking particular care not to neglect the non-pathogenic species which from a biological standpoint may be very important members of the group. Work on the anaerobes has often been limited by technical difficulties, the use of large quantities of media, elaborate apparatus, difficult sampling, danger of contamination, and lack of time and assistance. Simple test tube methods are much to be preferred and it is highly desirable that work on such a large and important group of organisms shall be brought within the facilities of

as many workers as possible. The work to be reported has been largely influenced by these considerations.

Material.

The material for the study consisted in strains from a number of sources. There were several strains from the collection of Dr. Theobald Smith, a strain of *C. putrificum* furnished by Prof. L. F. Rettger, a strain of *C. chauvoei* isolated from some "blackleg" muscle received from A. E. Hagan of the Kansas Blackleg Serum Company of Amarillo, Texas, a strain of *C. chauvoei* isolated from dried muscle, and one of *C. vibrion-septique* from similar material sent by Dr. W. A. Hagan of Cornell University, another strain of *C. vibrion-septique* isolated from some infected guinea pig muscle supplied by S. H. McNutt of Iowa State College, and a strain of *C. tetani* isolated at autopsy from the castration wound of a pig with clinical tetanus. Strain C. 3413 was from the feces of a cow, strains C. 710Fa, C. 710Fb, and C. 710Fe from the feces of another cow, and strain Clf. 993 from the pleural abscess of a calf. In addition to the above there were isolated as many different anaerobes as could be found in the normal intestinal tract of a calf slaughtered when one month old. These strains came from the contents of a loop of duodenum, a loop of ileum, some of the contents of the cecum, and some feces from the rectum. For the present report the source of these strains is regarded as unimportant. What was wanted was simply a representative collection of anaerobes from some source in nature. From the intestine of the calf there were studied three strains from the duodenum, three from the ileum, eleven from the cecum, and six from the rectum. If possible duplicates from each source are excluded; there were at least two distinct species from the duodenum, three from the ileum, seven from the cecum, and six from the rectum. From the entire intestinal tract of this animal there were isolated not less than fourteen different species. Most of those found in the cecum were present also in the rectum.

Methods.

The general plan of the study was as follows:

Thick suspensions of intestinal contents or fecal material were heated for fifteen minutes at 80°C. and then a drop or two of the

suspension was inoculated into vaseline tubes of fermented veal infusion bouillon, fermented bouillon plus 1 per cent of lactose, and unfermented bouillon plus 1 per cent of glucose. Each tube of medium contained a small piece of rabbit or guinea pig kidney placed in the tubes before they were autoclaved. After incubation for three days each of the cultures was heated for fifteen minutes at 80°C. By this method sporulating aerobes were eliminated (Brown, 1922). The cultures were then plated in horse blood agar in Petri dishes with clay tops glazed on the outside only and were incubated in the anaerobc jar (Brown, 1921). From the blood agar plates both deep and surface colonies were studied and as many different kinds as could be found were transplanted into veal infusion bouillon plus kidney tissue under vaseline. These cultures were studied and again plated on blood agar, the process being repeated until pure cultures were assured. No difficulty was experienced in obtaining and maintaining pure cultures and it is doubted whether the method of successive plating, careful study of the colonies, and fishing from deep colonies is any less dependable than that of picking out single cells or spores with a micro-pipette.

The strains isolated were studied in cooked meat medium under vaseline with reference to change in color of the meat, consistency, digestion, and formation of gas. The formation of a black ring of deposit at the surface of the medium was taken to indicate hydrogen sulfide formation. Cultures in cooked meat were the ones employed for study of the morphology of the organisms.

The growth of the colonies in deep agar shake cultures afforded means for studying the shape of deep colonies and gave a rough index of the relative degree of anaerobiosis favorable for the growth of each strain. In such tubes of agar the colonies grew to within from 3 to 15 mm. of the surface, the growth stopping abruptly at this level. Agar shake cultures also serve well as stock cultures and if a little sterile vaseline is pipetted onto the surface of the agar the medium keeps indefinitely, without drying and without contamination by molds.

Tubes of veal infusion gelatin under vaseline were inoculated in fluid condition and incubated with sterile control tubes at 35° to 37°C. for several weeks. From time to time they were taken from

the incubator and refrigerated or placed in cold water. The control tubes never failed to solidify. If the cultures failed to solidify, the gelatin was regarded as having been liquefied.

Cultures were grown in tubes of about 5 cc. of skim milk (tyndallized under vaseline) and also in citrated (transparent) milk (Brown and Howe, 1922). Gas formation, coagulation, and peptonization were observed.

The digestion of various protein substances was observed by placing small bits of the test substances in tubes of veal bouillon under vaseline. The proteins used were 5 mm. cubes of coagulated horse serum or egg white and small masses of rennet casein and of washed horse or calf fibrin. These substances were autoclaved in the bouillon.

The attempt to determine the ability of various anaerobes to ferment various carbohydrates led to a number of interesting observations. The carbohydrates were sterilized in 10 per cent aqueous solutions and added aseptically to tubes of sterile bouillon under vaseline. Each tube also contained a bit of rabbit or guinea pig kidney which had been autoclaved in the bouillon. The tissue was added to provide conditions for a good growth of the culture. Plain veal infusion bouillon was used rather than fermented bouillon because the unfermented bouillon is a much better medium for the growth of some strains and the small amount of muscle sugar present was not sufficient to be detected with Benedict's solution or to result in the formation of enough acid to inhibit the growth of the culture or to be confused with the fermentation of larger amounts of the test substance added. It was very soon discovered that the customary practice of adding 1 per cent of carbohydrate to the medium was not to be relied upon for determining the fermentation reactions of anaerobes. Certain strains were found to ferment as much as 3 per cent of glucose. Although in media containing an excess of carbohydrate the final hydrogen ion concentration usually gave distinct evidence of fermentation it did not always do so. Even in media containing 4 per cent of glucose (always an excess) some glucose fermenting strains occasionally reached a final hydrogen ion concentration near that produced by non-fermenting strains (e.g., Botulinum A, an active fermenter of glucose, reached a final reaction of pH 6.2, whereas strains IV, Cf and Ci₁, non-fermenters, also

reached reactions of pH 6.3, 6.2, and 6.3 respectively). The titratable acidity was found wholly unreliable as an index of fermentation. Certain strains incapable of fermenting glucose produced increases in titratable or reserve acidity of between 2 and 3 per cent normal acid (e.g., IV, Ca, Ci_1, and Dc). In such cases there was also a large increase in the reserve alkalinity, both combining to make a large increase in the buffer index (Brown, 1921). An attempt was made to find an indicator which might be added to the test media so that the changes in reaction of the cultures might be followed. Litmus, neutral red, china blue, and rosolic acid, reduced acid fuchsin, brom cresol purple, methyl red, phenol red, thymol blue and some others were tried but without exception all were decolorized by cultures of anaerobes whether fermentation occurred or not. Furthermore the color did not return to the indicators upon exposure of the culture to the air; they were permanently decolorized. Hall (1921) encountered the same difficulties in determining the fermentation reactions of anaerobes. Hall and Randall (1922) found cultures of *C. welchii* to reach a peak of acidity followed by a depression in the hydrogen ion concentration. Hall came to the conclusion that an increase in hydrogen ion concentration furnishes the best evidence of fermentation by anaerobes. To follow the changes in reaction that occurred in growing cultures of anaerobes a method for determining the hydrogen ion concentration of small amounts of fluid was devised (Brown, 1924). Peaks of acidity were noted to occur at different times in the case of different anaerobes. With *C. welchii* the peak occurred within 48 hours. With other anaerobes it might occur at the end of a week or two, sometimes later. The results were usually but not always clear cut. Defining fermentation as synonymous with saccharolysis it would seem that the best evidence of fermentation would be the disappearance of the test substance from the culture. We therefore adopted the method of adding only 0.2 per cent of the carbohydrate to the bouillon and after incubation for three months or longer testing for the presence or absence of the carbohydrate. Eight or 10 drops of bouillon containing 0.2 per cent of glucose, maltose, or lactose gave a distinct reaction with about 4 cc. of Benedict's solution. By adding 1 or 2 drops of dilute hydrochloric acid to 8 or 10 drops of the sucrose

bouillon and boiling it for a few seconds it also gave a positive reaction with Benedict's solution. The presence of starch was readily detected by the addition of Lugol's solution. If cultures in any of the above media were found to give negative chemical tests it was concluded that the carbohydrates had been fermented. Incubated controls always gave positive chemical tests. Manitol, glycerol, and salicin were also added to bouillon in a concentration of 0.2 per cent, but simple chemical tests for these substances were lacking. A biological test was therefore tried. A strain of the B. coli group was found to ferment these substances with gas production. Smith and Smith (1920) found that if strains of B. suipestifer were grown in lactose bouillon, B. coli was no longer able to form gas when superinoculated into these cultures, but that if the hog cholera culture in lactose bouillon was heated at or above 80°C. the gas inhibiting factor was removed and B. coli was able to form gas in the culture. Our method was as follows: After incubating the anaerobe cultures for a month or longer a determination of the hydrogen ion concentration was made and, if necessary, the reaction was adjusted to about pH 7.0 by the addition of alkali or acid. The culture was then heated in a boiling water bath for fifteen or twenty minutes, cooled, and superinoculated with B. coli. Good growth of the colon bacillus always occurred. Because of the high buffer index of many of the anaerobe cultures and the small amount of carbohydrate originally present (0.2 per cent) a marked increase in the hydrogen ion concentration due to fermentation by B. coli was not to be expected, but the formation of an appreciable amount of gas was interpreted as indicating the presence and fermentation of the test substance. B. coli in control tubes of medium in which anaerobes had not grown and which contained 0.2 per cent of test substance always produced considerable gas. On the other hand the absence of gas formation by B. coli in the old anaerobe cultures had to be interpreted with caution because in some anaerobe cultures containing glucose, maltose, sucrose, or lactose, as revealed by positive Benedict tests, B. coli failed to produce gas. We offer no explanation of the phenomenon but it would appear that the gas inhibiting factor is not always thermolabile as Smith and Smith found it to be in B. suipestifer cultures. In view of the difficulties encountered the results of the

fermentation tests for mannitol, glycerol, and salicin are not reported in this paper.

One other qualitative test requires explanation—the hydrolysis of milk fat. The ability of certain bacteria to hydrolyze fat has been noted by a number of authors referred to in the paper by Orcutt and Howe (1921). No systematic use seems to have been made of this differential character for the differentiation of anaerobes. For this purpose we have employed the cream of cow's milk. Thick cream was washed several times with salt solution and recovered by centrifugation. A thick emulsion of the washed cream was made in salt solution and sterilized in the autoclave. If this is done carefully the emulsion is not destroyed. About 0.5 cc. of this emulsion was then pipetted aseptically into 5 cc. of sterile bouillon under vaseline. In time the cream rises to the surface forming a compact layer 1 to 2 mm. thick just beneath the vaseline. Hydrolysis of the fat was manifested by more or less complete transformation of the cream into a thick (sometimes 10 mm. or more) layer of gray flocculent material sometimes sinking to the bottom of the medium when disturbed. Chemical analysis of this material by Dr. Paul E. Howe showed it to consist largely of fatty acids or soaps, doubtless depending upon the reaction of the culture fluid.

To compare some of the products of growth of the anaerobes in plain bouillon with those formed in the presence of an excess of glucose each strain was inoculated into the following media: a tube containing 5 cc. of veal bouillon plus 0.5 cc. of distilled water, and a tube of 5 cc. of the same lot of bouillon plus 0.5 cc. of a 40 per cent glucose solution. The water and the glucose solution were added aseptically. Each tube contained also a small bit of rabbit or guinea pig kidney to promote growth of the more delicately growing strains. The medium in each tube was covered by a layer of sterile vaseline 5 to 7 mm. thick. Sterile control tubes of each medium were incubated along with the inoculated tubes and were subjected to the same chemical analysis at the end of the period of incubation. The cultures were incubated for three months or longer if there was any evidence of continued activity. From time to time during this period the gas formed was measured and analyzed by the methods previously described (Brown, 1922). After each gas analysis the

vaseline seal was melted and allowed to fall upon the surface of the medium. An effort was made to compare the composition of the first gas formed with that given off later. Frequent determinations of hydrogen ion concentration were also made. At the end of the period of incubation a few drops of the plain bouillon culture were tested for indol by the dimethylaminobenzaldehyde reaction and a few drops of the glucose bouillon culture were tested for sugar with Benedict's reagent to make sure that there was still an excess of unfermented sugar present. From the contents of each tube there were also made buffer index (pH 5.0 to 8.0) titrations, formol titrations, and ammonia determinations. In the course of making these determinations there were also recorded the hydrogen ion concentration and the reserve or titratable acidity. The routine was as follows. Into each of four large (25 mm. diameter) test tubes there was accurately measured with an Ostwald pipette 1 cc. of the culture. To each tube was added 9 cc. of distilled water. Tubes I and II were used for the titration of the reserve acidity (R$_H$) to an end point of pH 8.0 and then for the formol titration (Brown, 1923). Tubes III and IV were of Pyrex glass and were used first for the titration of the reserve alkalinity (R$_{OH}$) to an end point of pH 5.0 and then for ammonia determinations. The latter were made as follows. To each tube were added 3 or 4 small quartz pebbles, a small amount of powdered talc, a couple of drops of mineral oil, and 1 cc. of potassium carbonate and potassium oxalate mixture (15 per cent of each). The ammonia was then distilled over a micro burner into dilute hydrochloric acid containing methyl red as an indicator. The unneutralized hydrochloric acid was titrated against N/20 sodium hydrate solution. The difference obtained by subtracting the ammonia from the formol titration is tabulated (table 3) as Amino Acids though it is to be understood as representing all formol titrating substances except the ammonia.

<div align="center">DISCUSSION OF RESULTS.</div>

The most conspicuous morphological feature of the anaerobes is the sporangium; the form and position of the spore and its size with respect to the cell. There are spherical spores and oval or elongated spores. The only strains with truly spherical spores encountered in

this study were *Tetanus S. 304* and *Tetanomorphum C. 3413*. The spores of these strains were of course terminal and in size several times the diameter of the bacillus. Next to these, morphologically, stood the strain of *C. putrificum* received from Dr. Rettger. This organism had an almost spherical terminal spore terminating a delicate slender bacillus. The oval spores of the remaining strains were placed terminally, sub-terminally, or excentrically. No truly centrally placed spores were found. The relative size or diameter of the spore with respect to the thickness of the bacillus bore a general relationship to the position of the spore. Without exception the terminal spores were relatively large and the bacilli were long, delicate, and slender. Sub-terminal spores were relatively a little smaller and the bacilli thicker. Excentrically placed spores were associated with rather thick rods, the spore sometimes expanding the cell very little. Of fifteen strains with terminal spores all were motile.

Hemolysis.—A majority of the strains studied produced beta hemolysis in the blood agar plate (Brown, 1919). The deep colonies were surrounded by clear, colorless, corpuscle-free zones. A minority of the strains were of the gamma type in blood agar, producing no visible change in the blood agar surrounding the colonies. None of the strains produced the alpha appearance. This is not surprising in view of the fact that the green-producing streptococci and pneumococci also fail to produce alpha zones under anaerobic conditions. Apparently oxygen is necessary for the production of the alpha zone. Strain Cf did produce a slight amount of greenish discoloration in the blood agar plate, but the green color appeared only after the anaerobe jar had been opened a second time for examination of the plates. It may be that if certain strains could be trained to grow aerobically they would produce alpha zones.

It is a striking fact that with the exception of *C. tetani* none of the terminal spore bearers produced hemolysis. It is especially to be noted that *Tetanomorphum C. 3413* was non-hemolytic. On the other hand all strains with sub-terminal or excentrically placed spores produced beta zones in blood agar, as did also *C. tetani*.

In the case of five strains, spores were not seen, although resistance to heat indicated that spores were produced by four of them. Of

these strains four produced beta hemolysis; three of these were iden-
tified as strains of *C. welchii* and one as *C. chauvoei* known to have
exccentric or sub-terminal spores. The remaining strain in which
spores were not seen was of the gamma type in blood agar, showing
under the microscope slender and actively motile bacilli—all char-
acters corresponding with those of terminal spore producers.

Colonies.—Two distinct types of deep colonies were noted, com-
pact biconvex colonies, and more or less diffuse rhizoid or woolly
colonies. The most reliable means of determining the form of the
deep colonies is in deep agar shake cultures after incubation for three
or four days. The blood agar plates were usually incubated forty-
eight hours. As this time the deep colonies of some strains were of
a conglomerate or compound type or biconvex with satellites, but in
deep agar shakes after longer periods of incubation these same strains
produced woolly or rhizoid colonies. Strains producing simple bi-
convex colonies in agar shake cultures always produced discrete round
or scalloped surface colonies on blood agar plates and were never
surface spreaders. Some of those producing woolly deep colonies also
produced discrete surface colonies but most of them produced rapidly
spreading amoeboid, rhizoid, or filamentous surface films. *C. tetani*
was one of the most persistent surface spreaders. The form of
colonies could not be correlated with motility, form, or position of
spore, or with hemolysis.

Staining.—All of the strains were Gram-positive when stained
with Stirling's gentian violet although some strains were not strongly
Gram-positive or were definitely Gram-positive only in young cul-
tures. Immature spores retained the Gram stain. Mature spores
were not stained. It was not uncommon to obtain Gram-positive
vegetative cells and Gram-negative sporangia containing Gram-
positive immature spores.

Lipolysis.—Fat digestion, though a definite property of many
strains, could not be correlated with any other character, morpholog-
ical or physiological. Certain lipolytic strains were of a fermenta-
tive character and others were predominantly proteolytic. Strains
which were both highly proteolytic and saccharolytic were also
lipolytic, but *C. tetani* which is neither highly proteolytic nor fer-
mentative was actively lipolytic whereas *Tetanomorphum C. 3413* was

not lipolytic.   Ability to hydrolyze fat is, therefore, a character which may be of considerable differential value within any large group of anaerobes, though it does not serve to distinguish one group from another.

Growth in Milk.- -Results obtained in skim milk were generally paralleled by those in transparent (citrated) milk except that in the case of one or two strains growth was a little more abundant and more apparent in the latter.   Reference to table 1 will show that milk was coagulated by most of the strains which failed to ferment lactose in bouillon.   The acidity reached by these milk cultures was between pH 6.2 and 6.8.   The coagulation in such cultures was a late coagulation, sometimes occurring during the second month of incubation.   The degree of acidity was hardly sufficient to cause coagulation.   On the other hand some of the whey from these cultures transferred to fresh raw milk failed to cause coagulation within two or three hours at 37°C. so that if an enzyme was the cause of coagulation it must have become inactivated by the time the test was made.

Biological Grouping of the Anaerobes.

All bacteria have a nitrogenous metabolism.   Most of them attack amino acids, polypeptides, peptones, and probably albumoses and to this extent may be said to be proteolytic, but some bacteria attack also higher native protein substances such as were used in our tests, i.e., gelatin, casein, serum, egg white, and fibrin.   The term proteolytic is usually reserved for those organisms which are able to attack the higher native protein substances.

Henry (1917) and others have grouped the anaerobes as saccharolytic, proteolytic, or as belonging to an intermediate group of organisms which are neither very proteolytic nor saccharolytic.   This grouping has been very useful, but most anaerobes are both proteolytic and saccharolytic and although one or the other activity may be the more conspicuous the other is not to be ignored.   The group of anaerobes we have studied, comprising many non-pathogenic as well as certain pathogenic strains, is probably more comprehensive biologically than would be a collection of strains from war wounds, and although this report does not include as many strains as might be

TABLE 1.

Growth in Cooked Meat and in Milk.

STRAINS	GROWTH IN COOKED MEAT							Gas	MILK		FERMENTATION OF LACTOSE
	Gas formed				Action on meat						
	Total gas	Per cent CO$_2$			Color	Diges-tion	H$_2$S		Coagu-lation	Pepton-ization	
		First	Median	Last							
I, Ff, Cc, Ce, Ch, Fe, Dd	0.1-0.6	4-24		23-28	Pink to red	-	-	0.0-2.0	+	-	+
Chauvoei (Denver)	0.3				Pink	-	-	0.0	-	-	+
Chauvoei (Texas)	tr.				Pink	-	-		No growth		+
Welchii X, Clf. 993, Ib, Ic	1.2-2.5	21-23		39-42	Pink to red	tr.	tr.	1.7-3.8	+	-	+
Putrificum	0.7	25	77	76	Pink to brown	+	+	Growth doubtful			+
Ci$_2$	1.3	66	97	68	Dark brown	+	+	0.6	+	+	+
Septique (Iowa)	0.5			24	Pink	-	-	0.2	++	-	+
Septique (Wash.)	0.2				Red	-	-	0.9	++	-	+
Cg	0.2			40	Light grey	+	±	0.4	+	+	-
III	1.6	40		99	Dark grey	++	++	0.6	++	++	±
II	1.6	85		99	Brown	++	++	1.1	++	++	+
Tetanomorphum (C. 3413)	0.2				Pink	-	tr.	0.0	-	-	-
Carno-foetidum	2.0	87	92	42	Brown to black	+	+	0.8	+	+	-
Sporogenes H, Botulinum A, Botulinum B, C. 710Fa, Fd	1.3-1.9	20-85	95-100	45-99	Brown to black	+	+	0.3-0.9	+	+	-

Group	First (vol.)	% CO₂ First	% CO₂ Median	% CO₂ Last	Color	First	Median	Last (vol.)			
Tetani S. 304	0.6				Pink	−	tr.	0.0	+	−	−
Fb	0.3	100		74	Grey to brown	+	+	0.4	+	+	−
Fa	0.3	88			Pink to brown	+	+	0.0	+	+	−
Cb, Ia, C. 710Fb	1.3–3.0	87	99–100	81–100	Brown	+	+	0.0–1.2	+	+	−
C. 710Fe, Fc, Ca, Histolyticum, Cd, IV	0.7–2.8	52–90	99–100	87–97	Red to brown	+	+	0.0–1.4	+	+	−
Ci, Cf, Da, Dc	0.0–0.5			83	Pink to brown	+	+	0.0	±	±	−

Explanation.—The transverse rulings serve to divide the strains into the "metabolic groups" defined in table 2.

Gas is measured in volumes. See explanation under table 3.

tr. = trace.

Under per cent CO_2: First = during the first part of the period of gas formation; Median = during the median period of gas formation; Last = at the end of the period of gas formation.

Figures separated by a dash (e.g., 0.1–0.6) indicate minima and maxima for the strains of the group.

desired it is believed that it furnishes the outline of a natural grouping which is illustrated in table 2. By noting the action of the anaerobes on a few protein substances and a few common carbohydrates there appears to be what E. C. Howe (1912) called a "metabolic gradient" with respect to each group of substances. Of the protein substances it is found that all the strains studied produced an increase in formol titration and in ammonia in pepton bouillon indicating that they attack amino acids and other low protein derivatives. Some strains could also liquefy gelatin; some could digest casein; some could digest coagulated albumin (egg white or serum); and others could also digest fibrin. Any strain which could digest fibrin was able to digest all the other substances in the series. Any that could digest coagulated albumin could also digest casein and gelatin. Any that attacked casein also liquefied gelatin. We have therefore a series of protein substances listed in the order of their digestibility by anaerobes. The carbohydrate series is not quite so rigid. The significant carbohydrates for determining the major groups are glucose, lactose, sucrose, and starch. Other substances may be useful in sub-dividing some of the major groups. Action on maltose always ran parallel to that on glucose. Glucose was naturally the lowest member of the series and was fermented by all strains capable of attacking any of the carbohydrates. Starch was the highest member of the series. Any strain fermenting starch was also able to ferment glucose and lactose or sucrose or both of the disaccharides. We did not encounter C. oedematiens or C. novyi which are said to ferment glucose and starch but neither lactose nor sucrose. In table 2, therefore, each strain listed is to be understood as attacking all the proteins listed to the left of its place on the chart but not those to the right, and all the carbohydrates beneath it unless otherwise indicated by a negative sign, but not those above it. For example, strain Cd attacks all of the protein substances but none of the carbohydrates, whereas Fe attacks all of the carbohydrates (except sucrose) but none of the proteins above proteoses. For descriptive convenience and accuracy we shall refer to the position of a strain in the table by naming the highest members of the carbohydrate and protein series which it attacks. For example, Fe is a starch-proteose organism, Cd a non-saccharolytic-fibrin strain. In a

rough sense, *C. vibrion-septique* may be said to belong to a "saccharo-lytic" group, *C. sporogenes* to a "proteolytic" group, and *C. tetani* to an "intermediate" group, but to which group does strain Ci₂ belong? It is more saccharolytic than *C. vibrion-septique* and as proteolytic as *C. sporogenes* and in both respects is unlike *C. tetani*. If, however, it is said that *C. vibrion-septique* belongs to the lactose-gelatin group, *C. sporogenes* to the glucose-fibrin group, *C. tetani* to the non-saccharolytic-gelatin group, and Ci₂ to the disaccharide-fibrin group, their metabolic positions are pretty well defined. If desired, many of the groups may be designated by the names of well known members.

The starch-proteose group is the *sphenoides-tertius* group.

The starch-gelatin group is the *chauvoei* group.

The starch-casein group is the *welchii* group.

The lactose-gelatin group is the *vibrion-septique* group.

The glucose-fibrin group is the *sporogenes-botulism* group.

The non-saccharolytic-gelatin group is the *tetanus* group.

The non-saccharolytic-fibrin group may be called the *histolyticus* group though it evidently contains two distinct sub-groups. To this group *C. putrificum* would also belong were it not for the fact that it has some action on carbohydrates. This species can not be dismissed as a mixed culture as was suggested by the British Medical Research Committee (1919). The strain received from Dr. Rettger had every appearance of being pure and a distinct entity. We plated it out many times. The morphology was exactly like the illustration in the paper of Reddish and Rettger (1922). By careful measurement the terminal spores were found to be almost but not quite spherical. The very slow delicate growth and the proteolytic activity were exactly as described by Reddish and Rettger. Although these authors describe *C. putrificum* as very slightly or not at all saccharolytic they did find that it was able to destroy an appreciable amount of glucose. In our study we have noted the disappearance of small amounts not only of glucose but also of lactose, sucrose, and starch, so that we regard the organism as having rather moderate but broad fermentative powers. In our grouping it belongs to the starch-fibrin group although its metabolism is carried on at a very slow rate. Cultures were sometimes incubated for 2 weeks before there

TABLE 2.

Grouping of the Anaerobes in Accordance with their Saccharolytic and Proteolytic Powers.

Saccharolytic group	Strain	Spore — Form and position	Motility	Form of deep colony	Type in blood agar	Lipolysis
	I	o., t.	+	c	γ	+
	Ff	o., t.	+	c	γ	+
	Cc	o., t.	+	c	γ	+
	Ce	o., t.	+	c	γ	−
	Ch	o., t.	+	c	γ	⧺
	Fe (sucrose −)	o., t.	+	c	γ	+
	Dd (sucrose)	o., t.	+	c	γ	⧺
Starch	Chauvoei (Denver)	o., exc.	±	c	β	+
	Chauvoei (Texas)		+	c	β	+
	Welchii X (capsules +)	o., exc.	−	c (capsules +)	β	⧺
	Clf. 993		−	c	β	+
	Ib (capsules +)		−	d	β	⧺
	Ic (capsules +)		−	d	β	+
	Putrificum	o., t.	+	d	γ	+
Sucrose and lactose	III (lactose ±)	o., s-t.	+	d	β	+
	II (sucrose ±)	o., s-t., exc.	−	d	β	+
	C₂	o., s-t., exc.	+	d	β	+
Sucrose or lactose	Septique (Iowa) (suc. −)	o., s-t., exc.	+	d	β	+
	Septique (Wash.) (suc. −)	o., s-t., exc.	+	d	β	+
	Cg (lactose −)	o., exc.	−	d	β	ſ

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	Glucose	Gelatin	Casein	Peptones or Proteoses	Carno-foetidum	Coagulated serum or egg	Fibrin			
		Tetanomorphum (C. 3413) r. t.	+	c	γ	−			Sporogenes H	o., s-t,	d	β	+
									Botulinum A	o., s-t., exc.	d	β	+
								+	Botulinum B	o. s-t., exc	d	β	+
									C. 710Fa	o. s-t., exc	d	β	+
								+	Fd	o., s-t.	d	β	+
		Tetani S. 304 r. t.	+	d	β	+	Fb	o., t.	+	C. 710Fe	o., s-t., exc	d	β	+
							Ia	o., t.		Fc	o., s-t., exc	d	β	−
						+			Cb	o., s-t., exc.	−		Ca	o., s-t., exc.	d	β	−
						+		Ia	o., s-t.	d	β	+	Histolyticum				
							C. 710Fb	o., s-t.		Cd	o., s-t.	d	β	−
								IV	o., s-t	d	β	+
								Ci	o., s-t	c	γ	±
								Cf	o., t.	c	γ	+
								Da	o.. t.	c	γ	+
								Dc	o., t.	c	γ	+

Proteolytic group — Non-saccharolytic

Explanation.— o. = oval; r. = round; t. = terminal; s-t. = sub-terminal; exc. = excentric; c. = compact, round or, biconvex deep colonies; d. = diffuse, rhizoid, or woolly deep colonies.

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was gross evidence of growth.   In one important respect our results differ from those of Reddish and Rettger.   We find *C. putrificum* a copious gas former if given sufficient time under the right conditions. The cultures were grown in vaseline tubes and the gas produced was measured in terms of the volume of medium.   The course of gas production in cooked meat (horse or beef heart) medium was as follows:

        Incubated   2 weeks—small bubble of gas
        Incubated   4 months—large bubble of gas, 25 per cent CO_2
        Incubated   7 months—0.7 volume of gas, 75 per cent CO_2
        Incubated 13 months—1.9 volume of gas, 79 per cent CO_2

Sub-cultures were made from the above culture at the end of four months and thirteen months of incubation and each of these went through the same slow course of development and gas formation. There was no evidence of contamination.   In plain bouillon plus a bit of guinea pig kidney 0.3 volume of gas was produced in four months and the final pH was 7.0, but in the same medium plus 4 per cent of glucose 2.8 volumes of gas were produced in four months and the final reaction was pH 5.9 (see table 3).   Gas formation was also increased and accelerated in bouillon containing maltose, lactose, sucrose, starch, mannitol, glycerol, and salicin.

With regard to other than saccharolytic and proteolytic characters some of the groups show a degree of uniformity which is encouraging to one who has frequently been discouraged by the apparent chaos among the sporulating anaerobes.   All the members of the starch-proteose group studied are slender motile bacilli producing oval terminal spores, and rather compact colonies of the gamma type in blood agar.   They differ with respect to the hydrolysis of fat. Members of the starch-gelatin and starch-casein groups produce beta hemolysis in blood agar, are non-motile (*C. welchii*) or slightly motile (*C. chauvoei*), produce sub-terminal or excentric oval spores, generally form compact colonies, and generally hydrolyze milk fat. Members of the glucose-fibrin group also produce beta hemolysis, have sub-terminal or excentric oval spores, are motile, form diffuse colonies, and actively hydrolyze milk fat.   Members of the non-saccharolytic-albumin group and the non-saccharolytic-fibrin group include at least two varieties, an oval terminal spore bearer, lipolytic

but non-hemolytic, and a sub-terminal spore bearer, non-lipolytic but hemolytic. All of the well known pathogens produce beta hemolysis in blood agar and hydrolyze milk fat. The morphological and gross cultural characters serve to group the strains as shown in table 2.

Biochemical Study.

A more detailed biochemical study was made of cultures grown in plain infusion bouillon and in the same medium plus 4 per cent of glucose, each tube containing also a bit of rabbit or guinea pig kidney (autoclaved) and a covering of vaseline. The biochemical results are tabulated in table 3. Although by the analytical methods employed duplicate determinations were found to give almost identical results when made with samples of the same culture, the results in table 3 are not to be read too closely. The biological factor is quite large and the metabolism of the anaerobic bacteria is very complex. If the same organism is grown in two or more tubes of the same medium, the results of chemical analysis may show not an actual divergence but considerable quantitative variation. The factors which influence the growth of the culture are not all understood or under control. It would appear that under certain conditions the proteolytic activity of an anaerobe may be most conspicuous, while under slightly different conditions the fermentative activity may be in the ascendency. It is necessary to interpret the results rather broadly and to emphasize general tendencies rather than minute differences.

Members of the starch-proteose group and those of the starch-casein group resembling *C. welchii* are readily recognized as fermenters of glucose. In 4 per cent glucose bouillon they produce a fairly high hydrogen ion concentration (pH 4.6 to 5.2) accompanied by a pronounced increase in reserve or titratable acidity and the evolution of a fair amount of gas. Botulinum A of the glucose-fibrin group produced a final hydrogen ion concentration of pH 6.2 in 4 per cent glucose bouillon, accompanied by only a fair increase in reserve acidity but a very large amount of gas. It consumed a large amount of glucose. Strain Cf also produced a final hydrogen ion concentration of pH 6.2 in 4 per cent glucose bouillon but the reserve acidity was very little above that of a plain bouillon culture and only 0.1 volume

TABLE III.

The Biochemical Analysis of Cultures in Plain Bouillon and in Glucose Bouillon.

STRAINS	FINAL pH	RESERVE ACIDITY Actual	Increase	BUFFER INDEX Actual	Increase	Formol titration	Ammonia	Amino acids	INDOL	Total	First	Median	Last	CO₂ Above the medium	In the medium	Total
I, Ff, Cc, Ce, Ch, Fe, Dd.	6.9-6.6	1.2-2.6	0.3-2.0	3.9-5.3	0.4-1 7	0.9-4.2	0.9-1.4	-0.3-3.3	‡	tr.-0.2	26-32		30-41	10.1-0.6	0 0-0.50 0	0 0-0.5
	5.2-4.7	3.8-6.4	2 7-5.4	3 8-6.8	0.1-2 9	-0.2-3 1	0 0-0 5	-0.5-3.1		0.3-1.6					0 0-0 2	0.1-0.8
Chauvoei (Denver)	7.0	1 0	0.1	4 0	0.5	0.6	1.9	-1.3	−	0.0	12		30	0.1	0.2	0.2
	6.0	3 2	1.1	4.0	0 1	1 0	1 0	0.0		0.5					0.2	0.3
Chauvoei (Texas)	7.1	1.7	0.8	6 6	3.1	4.2	6 2	-2.0	−	0.0				tr.	0.4	0.4
	6.0	3 2	1 2	4.4	0.5	0.2	1 3	-1.1		0.1					0.1	0.1+
Welchii X, Clf. 993, Ib, Ic.	7.3-6 8	1.2-1.4	0 3-0.7	4.1-4 7	7.1-1.2	1.6-6.1	2.0-2.4	-0.4-3.9	‡	0.2-0.3	36-41	47	40-51	0.90-1.1	0.2-0 40	2-0.4
	4.9-4 6	5.1-6 8	4.2-5.2	4 7-6.6	0.1-2.0	0.5-1.3	0.2-0 8	0 1-1.1		2.2-2.5					0-0.1	0.9-1.2
Putrificum	7.0	2.1	1.1	10.9	7.2	6.2	9.0	-2.8	‡	0.3	57		60	0.1	1.0	1.1
	5.9	3.6	1.7	7.2	3.0	4.6	5.0	-1.4		2.8				1.6	0.6	2.2
Cf3	7.0	1.5	0 7	8.2	4.7	4.9	6.9	-2.0	tr.	tr.	92	76	86	3.2	0.8	0.8
	5.1	4.8	2.8	7 1	3.1	4.4	2 0	2.4		3.7					0.5	3.7
Septique (Iowa)	7.1	1.5	0.6	5.0	1.5	1.8	1.3	0.5	−	0.2	34	42	36	tr.	0.3	0.3+
	5 6	5.4	3.4	6.5	2.6	5.3	1.5	3.8		2.1				0.8	0.2	1.0
Septique (Wash.)	7.0	1.2	0.4	6.8	3.3	2.7	4.9	-2.2	−	tr.	34	42	48	1.0	0.4	0.4
	5.5	4.5	2.5	5.5	1 6	2.6	1.3	1.3		2.5					0.2	1.2
C8	6.9	1.9	1.1	6 6	3.1	4 3	6.2	-1.9	+	tr.	57	66	57	1.8	0.4	0.4
	5.7	4.5	2.5	6 9	3 0	4.0	3.0	1.0		3.3					0.5	2.3
III.	7.2	1.3	0.6	8 2	5.1	9.8	8.4	1 4	tr.	0.1	61	89	63	0.1-	0.7	0.8
	5.5	4 2	2.5	6 0	2 3	6.7	3 7	3.0		5.7				4.5	0.0	4.5

Group	Strain																	
II	H	7.0 / 5.3	1.1 / 4.5	0.4 / 2.8	8.5 / 6.1	5.3 / 2.3	11.0 / 7.2	8.8 / 3.4	2.2 / 3.8	+	0.2 / 4.5	92	100	71	0.1 / 4.0	0.7 / 0.1	0.8 / 4.1
	Tetanomorphum (C. 3413)	6.9 / 5.7	1.3 / 3.6	0.3 / 1.9	5.6 / 5.5	1.9 / 1.6	1.3 / 0.9	0.5 / 0.5	0.8 / 0.4	−	tr. / 0.1			10		0.4 / 0.4	0.4 / 0.4
	Carno-foetidum	7.1 / 5.5	1.5 / 4.5	0.5 / 2.0	8.8 / 6.7	5.2 / 2.5	7.9 / 5.3	8.2 / 1.9	−0.3 / 3.4	+	0.0 / 4.1	71	94	81	3.6	1.1 / 0.5	1.1 / 4.1
	Sporogenes H, Botulinum A, Botulinum B, C. 710Fa, Fd	7.1–6 8 / 6 2–5.3	1.4–2.1 / 3.1–5.6	0 4–1.5 / 1.2–3.9	7 3–8.8 / 6 4–7.3	3.7–5 2 / 2.1–3.5	5.2–9.4 / 2.9–7.3	5 6–7.9 / 1.2–2 6	−2 0–2 3 / 0.5–5.1	−	0 0 tr. / 3.4–8.1	44–93	89–99	66–95	2.9–7.6	0.7–1.1 / 0.4–0.8	0.7–1.1 / 3.4–8.2
	Tetani S. 304	7.3 / 6 7	0.7 / 2 1	−0.1 / 1.0	6.7 / 8 0	3.2 / 4.1	3.1 / 5.9	4 8 / 5.0	−1.7 / 0.9	−	tr. / 0.2			50	0.1	0.8 / 1.3	0.8 / 1.4
	Fb	6.7 / 6.5	1 5 / 2 5	0 8 / 0 9	6.3 / 6.4	3 2 / 2.7	7.4 / 6.5	6.8 / 6.6	0 6 / −0.1	+	tr. / 0.1			55		0.5 / 0.6	0.5 / 0.6
	Fa	7.0 / 6.6	1.9 / 2.6	1.3 / 1.6	7.0 / 7 2	3 4 / 3 3	7.8 / 7.1	6 9 / 5.9	0.9 / 1 2	tr.	0 0 / 0.0					0.7 / 0.7	0.7 / 0.7
IV	Cb. Ia, C. 710Fb	7 0–6 7 / 7 0–6.6	1 8–1.9 / 2.3–3.1	0 9–1.1 / 0 7–1.9	7 8 / 7 6–8 7	4 1–4 7 / 3 8–4.9	6 3–11 / 8 6–10	37.0–11.2 / 07.6–9 9	−0 7–0.3 / 0 1–0 3	−	0.0–0.1 / tr.–0 2			40–60	0.1	0 6–0.7 / 0 6–0.7	0.6–0 7 / 0.7–0.8
	C. 710Fe, Fc, Ca, Histolyticum, Cd, IV	7 0–6 5 / 7 0–6 3	1 9–3.5 / 2 3–3.8	1 2–2.8 / 0.5–2.2	7 3–9 / 7.2–9.3	5 4 2–6.4 / 3.5–5 6	8.4–12 / 8.5–10.4	2 8.5–11 0 / 7.9– 9.2	−0 1–1.2 / −0.4–1.9	±	0.0–0 1 / tr.–0.3			40–76	0.1–0.2	0 6–0.8 / 0.5–0.8	0.6–0.8 / 0.6–1.8
	Ci, Cf, Da, Dc	7.0–6.7 / 6.9–6.2	1.2–0 3.7 / 2.5–3.7	1.1–2.8 / 1.3–3.2	7.8–8 9 / 7.4–8	4.3–6.4 / 6.3–6.5	5.2–10.4 / 4.0–10.5	8.6–13.5 / 6.8–10.1	−6 0–0.3 / −3.1–0.1	±	0.0–tr. / 0 0					0.4–0 7 / 0.5–0 7	0.4–0.7 / 5 0 7

Explanation.—The transverse rulings serve to divide the strains into the "metabolic groups" defined in table 2.

Figures separated by a dash (e.g., 1.2–2.6) indicate minima and maxima for the strains of the group. Figures in Roman type are for cultures in plain veal infusion bouillon. Figures in italics are for cultures in veal infusion bouillon plus four per cent of glucose.

The reserve acidity is the titratable acidity to an end point of pH 8.0.

The buffer index represents the amount of acid or alkali required to change the reaction of the culture from pH 5.0 to 8.0 or vice versa. The results of titration are in terms of per cent normal acid or alkali.

Figures under formol titration, ammonia, and amino acids indicate increases or decreases (when preceded by a minus sign) as compared with titrations of sterile incubated controls of the same media. The results are in terms of per cent normal. By multiplying these figures by 14 the results may be reduced to milligrams of nitrogen per 100 cc. of culture.

Amounts of gas above the medium and of carbon dioxide above and in the medium are expressed as volumes, e.g., 5 cc. of gas measured at atmospheric pressure and at incubator temperature, when given by 5 cc. of culture equals 1.0 volume of gas.

Under Per cent CO_2: First = during the first of the period of gas formation; Median = during the median period of gas formation; Last = at the end of the period of gas formation.

Numerical values of less than 0.1 are not reported.

of gas was produced. There was no appreciable consumption of glucose and all the available data indicate that this strain does not ferment glucose. Strains Ca and Ci_1 when grown in plain bouillon had a titratable acidity of 3.5 and 3.7 per cent normal respectively, or 2.8 above their sterile controls. However, their hydrogen ion concentrations were only pH 6.6 and 6.7 respectively, and in glucose bouillon they produced practically no gas and consumed no appreciable amount of sugar. The explanation of the high titratable acidities is to be found in the marked increase in buffer substances. They showed marked increases in reserve alkalinity as well as in reserve acidity, and the production of large amounts of ammonia indicated the deamination of large amounts of amino acids resulting in the liberation of fatty acids as well as ammonia. It is evident that proteolysis may result in an increased titratable acidity which may be mistakenly held to indicate fermentation. The results of Kendall, Day, and Walker (1922) illustrate the same phenomenon although they used titration as an index of fermentation.

Of the glucose fermenting organisms here reported Botulinum A produced the lowest final hydrogen ion concentration (pH 6.2) but the largest amount of gas (8.1 volumes) and in this culture consumed almost 4 per cent of glucose. Of the total gas produced 96 per cent was CO_2. A parallel culture of Botulinum B reached a hydrogen ion concentration of pH 5.3 and produced only 3.6 volumes of gas, of which 83 per cent was CO_2. Whether these differences are constant for other strains of Botulinum A and B has not been determined. It is noticeable, however, that all members of the sporogenes-botulinum group and the neighboring glucose-albumin group, when grown in glucose bouillon, consume large amounts of sugar, attain only a moderate degree of acidity (pH 5.3 to 6.2), and produce a large amount of gas (3.5 to 8.1 volumes), of which a large part (83 to 96 per cent) is CO_2. In contrast with this group is the tertius or starch-proteose group, which is almost purely fermentative in character. These organisms, when grown in glucose bouillon, actually consume less sugar than those of the sporogenes-botulism group but reach a higher acidity (pH 4.7 to 5.2) accompanied by less gas (0.3 to 1.6 volumes) of which only 26 to 36 per cent is CO_2. Evidently the percentage of CO_2 in the gas above the medium is not dependent

wholly upon the acidity of the culture. Various hypotheses suggest themselves as possible explanations of the differences. Members of the sporogenes-botulinum group are highly proteolytic. They produce marked increases in the formol titrating substances of the media. In plain bouillon a large part of the amino acids is deaminized. There is no apparent reason why the fatty acids liberated should not be further broken down with the liberation of gas. However, since very little gas was produced by any of the cultures in plain bouillon and since none of the non-fermenters produced more than a trace of gas, we must conclude that in glucose bouillon most or all of the gas is derived from the fermentation of the sugar. The large amount of gas produced by the sporogenes-botulinum group is doubtless related to their large sugar consumption. The large sugar consumption is permitted by the maintenance of a relatively low hydrogen ion concentration. The low hydrogen ion concentration is probably maintained by several factors; the marked increase in buffer index, largely the result of proteolysis; and the alkaline fermentation of organic acid salts as described for certain aerobic bacteria by Ayers and Rupp (1918). The formation of a high percentage of CO_2 by members of this group of anaerobes is strong evidence that the latter process is an important one. That proteolytic activity contributes to the same end, however, is indicated by the fact that if we compare the gas production of different proteolytic groups within the same fermentative group, the amount of gas produced and the percentage of CO_2 regularly increase with proteolysis; in fact by subtracting the volume of CO_2 from the total free gas produced it is found that the greater amount of total gas produced by the more proteolytic organisms is due entirely to CO_2.

Before we leave the study of gas production by the fermentative anaerobes another phenomenon is worthy of notice. The method of determining the CO_2 in the vaseline tubes made it possible to determine the percentage of CO_2 given off at various intervals during the period of gas formation. This was done in the case of glucose bouillon cultures and cooked meat cultures. In all cases it was found that the first gas given off contained a lower percentage of CO_2 than that given off during the middle period of gas formation, (noted also by Wolf and Harris, 1917, for *C. welchii*, and by Bushnell, 1922,

for *C. sporogenes*), and in most cases the last gas given off also contained a lower proportion of CO_2. The increase in CO_2 may be the result of a rising acidity but the subsequent drop in the percentage of CO_2 could not be explained by a change in reaction because in 4 per cent glucose bouillon a reversal of reaction did not occur.

It is not surprising that any of the fermenters of glucose should produce gas in cooked meat medium (see table 1), but it is surprising to find that the largest amounts of gas in this medium were produced by strains (e.g., Histolyticum Fc, Ca, Cb, Cd, and Ia) which did not ferment glucose or any of the test carbohydrates used. The same strains did not produce more than a few bubbles of gas in plain bouillon or glucose bouillon but produced gas in milk and gelatin as

TABLE 4.

Gas Production by Non-Fermentative Anaerobes.

STRAIN	MEDIA				
	Plain bouillon	Glucose bouillon	Cooked meat	Milk	Gelatin
Fc	Trace	0.12	1.69	1.4	1.4
Ca.................................	0.1	0.1	2.05	0.9	0
Cb	0	0.1	3.0	1.25	0
Cd.................................	Trace	0 1	2.81	0.9	1.8
Ia..................................	0.1	0.17	1.5	Trace	Trace
Histolyticum.......................	Trace	Trace	1.0	0	2.0

Figures are in terms of the volume of medium used.

indicated in table 4. Less gas was produced in transparent citrated milk than in plain skim milk. In all media the gas produced by these strains during the middle of the period of gas formation was 100 per cent CO_2. It is difficult to account for the gas formation by these non-fermenters of glucose unless they are able to attack certain lower carbohydrates or organic acids which may be present in these media but are absent from infusion bouillon. The acidity did not rise above pH 6.5 and in most cases was near pH 7.0.

The amount of CO_2 in solution in the media revealed nothing of recognized importance. All bacteria probably produce some CO_2; the result of what Hesse (1893) called the respiratory activity of bacteria. The amount of CO_2 found in solution bears a fairly con-

sistent relation to (*a*) the amount of growth, (*b*) the hydrogen ion concentration of the culture fluid, and (*c*) the CO_2 tension of the gas above the medium. The amounts of CO_2 found in cultures of the anaerobes in plain and glucose bouillon are tabulated in table 3.

Differences in the metabolism of the non-saccharolytic and the saccharolytic anaerobes are quite evident. The non-fermenters produced very little gas in either plain or glucose bouillon. Although the hydrogen ion concentration of the glucose bouillon cultures was never above pH 6.2 it was usually a little higher than that of the plain bouillon cultures. This may be attributed in part to the fact that the incubated sterile control tubes of glucose bouillon were usually a little more acid than the plain bouillon.

The buffer indices of the more proteolytic anaerobes were higher than those of the less proteolytic strains. As might be expected, the formol titration usually ran roughly parallel with increase in buffer substance. The amount of NH_3 produced is an index of deamination but is not necessarily a measure of proteolysis as has been assumed by Kendall. The proteolytic process may progress as far as the production of amino acids without giving evidence in the form of increase in ammonia. Strain Fe (table 3) shows a tendency to do this. On the other hand the amino acids alone do not furnish a good index of proteolysis, as was assumed by DeBord (1923), because they may be more or less completely deaminized as was the case with all the non-fermentative anaerobes studied. However, the formol titration which includes ammonia, amino acids, and the lower polypeptides is a very good index of proteolysis when any of these products are formed. The figure for the total ammonia determination was naturally never as large as that for the formol titration but the increase in the ammonia was often greater than the increase in the formol titration and could only indicate a decrease in amino acids and similar substances (e.g., Strains Cf and Ci_1).

In the case of the fermentative anaerobes generally the increase in formol titration of the plain bouillon culture was greater than that of the glucose bouillon culture, suggesting that the carbohydrate had a sparing action on the protein to some extent, sometimes greater than if the ammonia alone were taken as an index of proteolysis (Ic, I, Fe, Ff, Cc, Ce, Dd, and Ib) but more often less (Ch. *C. welchii*

X, Clf. 993, *C. chauvoei* Texas, Ci_1, *C. vibrion-septique* Washington, Cg, III, *C. sporogenes, Carno-foetidus,* II, and *Botulinum B*). In the latter cases one might say that the carbohydrate had spared amino acids to a greater extent than it had spared the protein. However, in the case of strains *C. vibrion-septique* Iowa, *Botulinum A* and *C. chauvoei* Denver, it might be concluded that the carbohydrate had stimulated proteolysis since the formol titration of the glucose bouillon cultures showed a greater increase than that of the plain bouillon cultures. We have also experimented with the effect of an excess of glucose on the digestion of certain protein substances by a saccharo-proteolytic anaerobe. *Botulinum B* was used for this purpose because it appeared to show a marked protein sparing action by glucose. The digestion of coagulated serum and of cooked meat was noticeably less in the presence of glucose, but the liquefaction of gelatin was not prevented. Other anaerobes gave similar results. All of the above results, however, must be interpreted with caution. If it were possible to study the metabolism of a single bacillus or of a constant number of active bacilli the meaning of the results might be quite clear, but in bacterial cultures there are the complicating factors of growth and activity. The accumulation of acids or other inhibitory substances may be responsible for the reduced nitrogenous metabolism. A glucose bouillon culture of a fermenting organism generally has the appearance of heavier growth than a plain bouillon culture but it may show a more rapid development and shorter life than the plain bouillon culture. It may be that the proteolytic activity of these cultures is proportional to the total amount of bacterial growth and activity and that the sparing action of the carbohydrate is only apparent. The problem calls for more careful control of all the possible factors involved.

<div align="center">SUMMARY.</div>

No markedly pathogenic or toxin producing anaerobes were found in the intestinal tract of the calf studied.

Both proteolytic and saccharolytic anaerobes were found at all levels of the intestinal tract but the putrefactive organisms were more abundant in the cecum and large intestine.

Of all the strains studied from the calf and from various other

sources, none produced the alpha appearance in blood agar, a large majority produced beta hemolysis, and some produced the gamma appearance.

More than half the strains studied had the ability to hydrolyze the fat of cow's milk to soaps or fatty acids.

By noting the action of anaerobes on a few protein and carbohydrate substances it is found that each of these groups of substances may be arranged in a series in order of the ease with which the substances are attacked by anaerobes. Strains which attack the same carbohydrates and the same protein substances form "metabolic groups." Within each group there is a fairly satisfactory correlation of morphological, physiological, and pathogenic characters so that the groups form the basis of a convenient and natural laboratory classification.

Some anaerobes produce profound changes in media within a few days. Some may complete their fermentative activity within a few days while their proteolytic activity may extend over several months. Others may produce perceptible fermentation or proteolysis only after two or more weeks and the activity may continue for several months. Whether the changes produced throughout these longer periods are due to actively growing organisms or to enzymes elaborated and released into the media has not been determined.

The titratable acidity is wholly unreliable for determining the occurrence or non-occurrence of fermentation by anaerobes. These organisms often produce large increases in the buffer index of media. The increase in titratable acidity is not necessarily accompanied by increase in hydrogen ion concentration and may occur in the absence of fermentation. In the presence of an excess of carbohydrate fermentation is usually indicated by an increase in hydrogen ion concentration but for many strains 1 per cent of carbohydrate is insufficient. A few cultures have been found to ferment as much as 3 per cent of glucose and arrive at a hydrogen ion concentration only a little above neutrality. The disappearance of the carbohydrate from the medium is regarded as the best evidence of fermentation.

The chemical analysis of cultures grown in plain bouillon and in bouillon plus 4 per cent of glucose (always an excess) has shown that neither ammonia production nor amino acid production is a good

index of proteolysis but that the formol titration provides a much better index.

The results of gas measurement show that much more gas is produced, though more slowly, by some of the more proteolytic anaerobes than by *C. welchii*. Gas analysis shows that the proportion of CO_2 to other gases found above the medium is least at the beginning of gas formation, increases often to 100 per cent of the gas given off during the intermediate period, and again decreases towards the end of the gas forming period. Whether the gas produced by anaerobes is all from carbohydrates or not remains an open question.

Although in cultures of anaerobes there is usually less proteolysis in the presence of an excess of fermentable carbohydrate many other factors must be taken into consideration before it can be said that the carbohydrate has "spared" the protein in the sense that it has altered the metabolism of the organisms.

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THE RELATION OF BACILLUS ABORTUS FROM BOVINE SOURCES TO MALTA FEVER.

BY THEOBALD SMITH, M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

The existence of races indistinguishable fundamentally and differing only in minor physiological characters has presented a major problem to comparative pathologists since the methods of Koch have come into use. The paratyphoid, the tubercle bacillus, and the streptococci are well known examples. To this group must now be added the *melitensis-abortus* species. The races of tubercle bacilli have definite hosts and this fact is of great value in preventing confusion. The paratyphoid races, undoubtedly originating or developing in different host species, have not yet been definitely referred to their respective hosts. The *abortus* species also has definite hosts to which the races may be referred, the cow, the goat, and the pig. Perhaps races may be found in the future allied with other animals.

The practical significance of these host relationships bears upon the occasional invasion of other hosts. Statements based on recent comparative studies of *B. abortus* and *B. melitensis* have been made to the effect that these two organisms are identical and that a clinical complex similar to Malta or undulant fever may be produced in man by *B. abortus*. On the other hand, the well defined geographical limitations of Malta fever and its relation to goat's milk, the high degree of infectiousness of *B. melitensis* towards laboratory workers handling this organism, and the wide diffusion of infectious abortion in cattle throughout Western Europe and the United States, all militate against this identification. They make it desirable to analyze more minutely any differences that may exist between these races and to trace more definitely the source of infection in the few sporadic cases of Malta fever in man occurring in regions where goat's milk and its products do not openly appear in the markets.

The close relationship between *B. abortus* and *B. melitensis* in morphological, biological, and serological characters was first pointed out by Alice C. Evans in 1918 (1). Although *B. abortus* was discovered by Bang in 1897 and *B. melitensis* by Bruce in 1891, and although the fundamental relation between these organisms is such that it could hardly have been overlooked had the two organisms come under the observation of the same person, the bringing together had to wait many years because of the hitherto separate flow of research in human and animal diseases. The essential identity of the two organisms pointed out by Evans has been affirmed by all those who have subsequently compared them. The problem to be solved concerns the minor differences which make one pathogenic for man, the other not, except perhaps under unusual conditions still to be defined.

Besides the use of immunological reactions in a comparative study of races or varieties the use of animals may be of decisive importance, as was shown by the writer in distinguishing between mammalian races of the tubercle bacillus. The problem is to choose an animal in which differences, if such exist, are most highly magnified and brought out regularly and without much variation.

Fleischner, Vecki, Shaw, and Meyer (2) carried out a number of experiments on several species of monkeys with *B. abortus* and *B. melitensis*. They used intravenous inoculation and feeding in milk and on solid foods. They conclude that although virulent strains of *B. abortus* may be pathogenic for monkeys, *B. melitensis* is far more invasive.

Burnet and Lagoanère (3) inoculated rats and mice with both organisms but they were unable to find any differences. Durham (4) attempted to produce a fatal disease in guinea pigs by injecting a large dose into the cerebrum and raise its virulence by passages.

Eyre (5) describes an acute and a chronic disease in guinea pigs resulting from intracerebral inoculations. In the chronic disease the animal is distinctly ill for 3 to 6 days but gradually recovers. After an interval of weeks or months during which the animal appears in perfect health, death suddenly takes place. No characteristic lesions are described by Eyre. The urine was usually infected.

Nicolle and Conseil (6) describe a spontaneous infection of guinea pigs with *B. melitensis* in a goat stable in Tunis. Somewhat later the same authors (7) inoculated two guinea pigs and fed two others with a culture of *B. melitensis* recently isolated from a goat. One of each pair was killed on the 42nd day. *B. melitensis* was isolated from spleen, blood, bile, but not from liver. The urine

was not examined. The other pair was killed on the 134th day. There was slight hypertrophy of the spleen. Cultures were obtained from spleen and urine.

In 1911, the writer and Fabyan (8, 9) first described a slow, progressive disease in guinea pigs following the injection of *B. abortus* and having quite characteristic lesions. Since that date, guinea pigs have been used quite regularly for the isolation of *B. abortus* from uterine fluids and milk containing a variety of bacteria.

Khaled (10) states that ¾ of a slope of *B. melitensis* killed a guinea pig of 240 gm. in 18 hours. To kill a guinea pig of 240 gm. in approximately the same time, 4 ½ slopes of *B. abortus* were required, thus showing that *B. melitensis* is about 6 times as virulent as *B. abortus*. A history of the cultures is not given.

K. F. Meyer, Shaw, and Fleischner (11) studied comparatively the lesions produced by *B. abortus* and *B. melitensis* in guinea pigs. The method used was to inject the cultures directly into the testicles, the dose being 1/5 to 1/20 of an agar slant, or from 1 to 2 billion bacteria. The results with *B. melitensis* were that "four cultures . . . . infected guinea pigs quite regularly, while eighteen other strains either proved non-pathogenic or occasionally produced lesions in the spleen, lymph nodes, etc." The disease produced by *B. abortus* was either acute or chronic and varied from strain to strain. It involved the spleen, lymph nodes, testicles, and joints. They conclude that "*B. abortus* is as a rule slightly more invasive and virulent than *B. melitensis*."

Jaffé (12) in the same year published results of similar import. He injected strains of *B. abortus* and *B. melitensis* into the testicles, ovaries, the peritoneal cavity, and the subcutis. His cultures, four of each race, came from Kral's collection. The dose used was 1/10 of an agar culture. Jaffé finds the lesions caused by both races much the same, although those due to *B. abortus* were more pronounced.

Et. Burnet (13) in discussing the lesions in guinea pigs due to inoculation with *B. melitensis* mentions hypertrophy of spleen and invasion of the marrow of the long bones. 5 per cent of the inoculated animals developed paraplegia. The histological changes are not discussed. Jaffé states that endothelial foci are produced by *B. abortus* and *B. melitensis* and that necrosis does not occur either in testicles or spleen. K. F. Meyer, Shaw, and Fleischner (11) describe the lesions due to *B. melitensis* as "nests of epithelioidal and scattered giant cells . . . . common to all focal lesions in the lymph nodes, spleen, and liver. . . . . The pathologic process is indistinguishable from that seen in guinea pigs affected with *B. abortus*."

Bearing upon the relation between *B. abortus* and *B. melitensis* the experiments of Nicolle, Burnet, and Conseil (14) are of interest. They injected subcutaneously 800 to 900 million living bacilli from 24 hour cultures of *B. abortus* into five human beings. No fever or other troublesome symptoms followed. They suggest the use of *B. abortus* in treating Malta fever.

Polettini (15) has recently described an anaphylactic test which is to distinguish between the bovine and the caprine race. The dried and powdered growth from agar cultures is suspended in a 1 per cent solution of anhydrous sodium carbonate

in distilled water. Rabbits received into an ear vein 1 to 5 cg. of the suspended powder of either strain. There was no change during ½ hour's observation of the blood pressure, respiration, or coagulability of the blood. They then received seven injections of the same dose subcutaneously 1 week apart. 12 days after the last injection they received 2 cg. into an ear vein. When *B. melitensis* followed treatment with *B. abortus* there was noticed within 1 ½ minutes after intravenous injection a fall in blood pressure from 180 to 130 mm., rise in respiration to 100 per minute, and coagulation of the blood in 60 minutes. These changes did not occur when *B. abortus* followed treatment with *B. abortus*.

Assuming that the observers failed to present any salient distinguishing characters between *B. abortus* and *B. melitensis*, the writer considered the subject still open. Two conditions probably interfered with clear-cut results. One is the use of old cultures, and the other the injection of too large doses. The inoculation disease in guinea pigs is a slow, chronic process and has many characters like tuberculosis. All who have worked with tubercle bacilli know that the acute disease following large doses is very different from the chronic type due to minute doses. It is the latter type which is of value in the recognition of races and varieties.

The desirability of determining more definitely the relation between *B. abortus* isolated from cattle and the races isolated from human subjects manifesting certain well defined symptoms which are here called Malta fever for convenience is emphasized by the great economic importance of *B. abortus* in the dairy industries and the serious situation which might be created if it were assumed that *B. abortus* produces a diseased condition in man. Fortunately a human culture recently isolated in New Haven and kindly offered by Dr. Francis G. Blake was available for a comparative study. This strain is designated *B. melitensis* II. Two separate cultures from the same patient were tested as II$_a$ and II$_b$ and found identical. At the same time an older culture isolated from a case in Baltimore and described by Keefer (16) was drawn into the comparative study through the kindness of Dr. Harold L. Amoss. This strain is designated *B. melitensis* III. It was isolated in 1922.

Although hundreds of strains of *B. abortus* have been isolated and passed through guinea pigs in this laboratory since 1917, it was deemed best to associate with these two strains of *B. melitensis* two

of *B. abortus*, both isolated through guinea pigs in September, 1924. One (1114) had a high CO_2 requirement, *i.e.* it multiplied very slowly in sealed tubes and not at all in open tubes, while the other (1119) was far less exacting and multiplied in unsealed tubes but only in low dilutions.

The chief characteristic features of the *B. abortus* disease in guinea pigs having been described some years ago (8, 9) it is in order to summarize them briefly, in view of a further prolonged study of them since 1917.

In guinea pigs receiving minute doses of recently isolated strains or material from bovine uterus or fetus the course of the disease and the resulting lesions are quite uniform in character. Usually there is a slight retardation in the normal weight curve during the 1st and 2nd weeks. Thereafter the weight advances as in the normal animal. There is no local swelling, abscess, or ulcer and only a slight enlargement of the regional lymph nodes.

Information obtained from guinea pigs killed at different periods following inoculation brings out a certain progression in the lesions observed. The spleen begins to enlarge in the 2nd week and continues up to the 8th or 10th week. The enlargement is mainly a congestion due to the distension of the sinuses and following the appearance of endothelial foci. The organ becomes very rich in blood. Minute grayish foci appear, often in large numbers, which rarely exceed 1 mm. in diameter and remain grayish and firm. These are often concealed by the congestion. The linear spleen dimensions may be three times the normal. When the congestion remains slight, the surface of the organ is no longer smooth but slightly nodular.

After the 4th week minute, yellowish depressions dotting the liver surface appear. They may be very few or fairly numerous. The subcutaneous lymph nodes become swollen but remain inconspicuously buried in the local fat deposits and do not show any distinct macroscopic changes.

After the end of the 2nd month other localizations may appear. The kidneys become pale, or even white, and enlarged, due to interstitial foci coalescing and leading to a slow destruction of the cortical tissue. The carpus or tarsus may be swollen, ribs enlarged and rarefied. The ciliary region of the eyes may become infiltrated and paralyses due to localizations in the spine and membranes of the cord may appear quite suddenly. These special localizations were far more frequent in the material from Massachusetts studied in 1910 and 1911 than in the more recent investigations from 1917 on. There is here presented the possibility of a gradual modification and attenuation of the virus in the bovine species during this period, or else the regional occurrence of types slightly differing from one another.

Lesions of the testicles are rarely absent after the 2nd month and they may

appear soon after the 1st. The epididymis is the chief site. The interstitial tissue becomes infiltrated and it compresses and obliterates the tubules. The entire focus becomes permeated with polymorphs and converted into a cheesy mass. The dominant lesion is an infiltration of spleen and lymph nodes with cells of endothelial type, forming roundish tubercles or more diffuse meshworks.

B. abortus is present in the spleen in largest numbers during the 3rd and 4th weeks (17). After the 8th week, the spleen may be very large but the bacilli scarce. Cultures may fail at this stage if old strains have been injected. They usually fail unless bits of spleen of pea-size, or even larger, are introduced into the culture tubes.

As stated above, the disease in guinea pigs resembles in many respects tuberculosis in the same species, excepting that the tubercular guinea pig dies sooner or later whereas the *abortus* guinea pig recovers, or more accurately does not become visibly ill at all except for occasional localizations in the bones, the eyes, the cord, and the kidneys. Death has been observed as a result of paralysis and of rupture of the enlarged and congested spleen.

EXPERIMENTAL DATA.

Two recently isolated strains of *B. abortus* were inoculated into guinea pigs in the same doses used in studying the effects of the two strains of *B. melitensis*.

To prepare the culture for injection, a fresh agar slant was inoculated, sealed, and allowed to develop into a uniform grayish film which usually reached its height in 48 to 72 hours. The sealing tends to insure uniformity of growth in the cultures to be compared. If tubes are left open, strains not requiring CO_2 grow more richly than recently isolated *B. abortus* strains. The seal restrains the former and assists the latter. The resulting films are therefore much the same quantitatively. The tubes used measured about ⅝ inch and contained 6 cc. of nutrient agar. The sealing is done with sealing wax and requires care since even minute breaks modify the growth.

The film is washed down with the condensation water. This was about 1 cm. deep and ⅓ cc. in volume. The condensation water becomes heavily turbid and contains the entire mass of bacilli. This was diluted twice in 10 cc. normal saline with the aid of a platinum loop holding about 0.004 cc. The second dilution contained in 1 cc., 0.00000016 cc., or $\frac{1}{6,250,000}$ cc. of the condensation water. Multiplying this figure by 3, we obtain the dilution of the entire culture suspension since the condensation water measures only ⅓ cc.

The guinea pigs received either ½ cc. or 1 cc. into the subcutis or peritoneal cavity. Twenty-one guinea pigs, in all, were inoculated

with the minute doses of *B. melitensis* II and III and killed at intervals beginning with the end of the 3rd week and ending with the 11th week. All animals survived except one which died following a rupture of the diaphragm due to some accident. An equal number were inoculated with the same amounts of *B. abortus*, Strains 1114 and 1119. Besides these a number of guinea pigs inoculated with fresh material from the bovine disease received during the investigation were included in the study.

The course of the disease due to the two strains of *B. melitensis* was much the same. *B. melitensis* II was, however, more virulent in that the lesions were more extensive. This may perhaps be explained by the fact that *B. melitensis* III had been under cultivation about 2 years when the present inoculations were made, whereas *B. melitensis* II was only 4 months old.

Comparing the changes in weight of the two groups—*B. melitensis* on the one hand, *B. abortus* on the other—the average gain of the former in a period of 5 weeks was about 100 gm. per animal, that of the *B. abortus* group 200 gm. After 9 weeks the average gain of the eight *B. abortus* animals was 300 gm., that of the seven surviving *B. melitensis* animals 197 gm. The average gain of the *B. melitensis* group II was 103 gm., that of the *B. melitensis* group III 242 gm.

The mode of inoculation, whether into subcutis or peritoneal cavity, determined to a certain degree the extent and age of the lesions in the lymph nodes. The subcutaneous inoculation usually produced suppurating kneefold nodes up to 1 cm. in diameter. After 9 or 10 weeks these were mere sacs filled with a cheesy mass which emerged from the incision under pressure as a tape-like mass. The involvement of the other superficial lymph nodes varied from animal to animal. The most frequently attacked nodes were the cervical lymph nodes and the thymus, next the axillary. In these glands there was a variable number of roundish, yellowish foci from 1 to 3 mm. in diameter. In one animal both thymus glands were the seat of foci 1 cm. in diameter. In all cases the foci were centrally softened or cheesy after 4 weeks. The bronchial node at the apex of the thorax became swollen later, but necrotic foci were not observed.

The spleen during the early weeks became enlarged and congested

and resembled closely the *B. abortus* spleen. In some animals it was permeated with large numbers of minute grayish foci. Later a smaller number enlarged and took on the appearance and consistency of the lymph node foci described above.

The lesions of the testicles were scarcely distinguishable from those of the *B. abortus* disease, because in both the localization is the same and softening of the foci occurs after a time. The lesions due to *B. melitensis* were in some cases associated with extensive edema, probably because of involvement of the pelvic lymph nodes. The contents of the seminal vesicles became clouded and opaque due to suppurative infiltration, a condition seen up to the present in only one *B. abortus* animal. In both groups, minute, depressed, grayish or yellowish point-like foci were present in the liver.

In the guinea pigs kept for 10 or 11 weeks, one out of eight *B. abortus* animals developed a swollen carpal joint. Of the eight *B. melitensis* guinea pigs, one had a swollen tarsal joint and one paralysis of the hind limbs.

The histological character of the lesions is best seen in the lymph nodes. The foci of endothelial elements cause a rarefaction of the greater portion of the gland tissue on account of the poverty in chromatin of the nuclei and the feebleness or absence of any cytoplasmic stain. On the periphery of the nodes there is still a narrow zone of normal lymphocyte elements. The changes which the neoplastic areas undergo in *B. melitensis* infection after the 3rd week are limited to foci not smaller than 0.6 mm. in diameter. These become centrally occupied by polymorphs which form a distinct nucleus. The larger foci or those areas formed by the union of several foci may undergo central necrosis either before or after invasion by polymorphs. In the latter case the necrotic central mass is densely filled with nuclear debris of polymorphs. In the former case the necrotic center contains but little nuclear debris and it is surrounded by a demarcating wall of partly necrotic polymorphs.

Liver foci in the guinea pigs inoculated with the bovine cultures remain very small. They are not more than 1 mm. in diameter, usually slightly depressed and of a yellowish color. They are not very characteristic histologically and are evidently metastases from the spleen leading to necrosis of a few liver cells and subsequent

infiltration with lymphocytes and a few endothelial elements. In the guinea pigs inoculated with *B. melitensis* liver foci appear and later on tend to enlarge and become centrally softened.

B. abortus in Swine.—In view of the results obtained it seemed necessary to look elsewhere for the source of the human infections. The existence of a race of *B. abortus* causing abortion in swine was first pointed out by Good and Smith in 1916 (18). In 1914 Traum[1] had isolated *B. abortus* from the organs of an aborted fetus. Since then the existence of infectious abortion in swine has been observed by others in the Middle West. The bacillus isolated resembled the bovine type and agreed with it serologically. It grew, however, in unsealed cotton-plugged tubes from the start. A number of investigations of swine abortion have been published since 1916. From the material there presented, two problems appear; first, the significance of the porcine race in the abortion disease of cattle and the reverse, and second, the nature of the guinea pig disease induced by the porcine race. Taking up the published experiments on the first problem, the identity of bovine and porcine races, we find the following data at hand.

Hadley and Beach (19) produced abortion in a pregnant cow and in sows with the porcine strain. A bovine strain caused abortion in a pregnant cow, but not in sows. Cotton (20) failed to infect pregnant sows with bovine strains by feeding. Porcine strains failed in two pregnant cows after intravenous injection. In another cow treated similarly, premature birth followed. The fetus and placenta were infected.

Huddleson (21) fed three virgin pigs with naturally infected milk and virulent cultures of bovine origin, beginning when pigs were 8 weeks old and continuing to time of farrowing, without producing abortion. Four pigs placed with infected cows remained normal. Six fed large doses both of bovine and porcine cultures repeatedly, dropped normal litters.

Schroeder and Cotton (22) produced abortion in pregnant cows by intravenous injection of swine abortion cultures, but not by feeding or cohabitation of swine-infected cows with other cows. Sows were not infected by feeding bovine strains.

Before dismissing these experiments of direct feeding or inoculation of cattle and pigs with homologous and heterologous strains, it may be stated that intravenous injection of pregnant animals may

[1] Quoted by Hayes (23).

break down a resistance sufficient to prevent infection through natural agencies. On the whole the experiments quoted are not conclusive. They indicate either that the porcine type of bacillus is harmless to cattle and the reverse, or else that the porcine type is of higher virulence and may infect cattle.

The effect of *B. abortus* from swine on guinea pigs is mentioned by several writers.

Hayes (23) states that "porcine strains proved, on the whole, to be more virulent for guinea pigs, causing, with a few exceptions, involvement of the testes and one or both radiocarpal regions, also general adenitis. Thirteen of twenty-two guinea pigs inoculated with porcine strains succumbed within 2 months, while none of the twelve inoculated with the bovine strain died of infection during that period."

Cotton (20) noticed differences between the lesions in guinea pigs produced by the swine abortion strain and the bovine strains. The lymph nodes are "converted into sacs filled with semi-fluid, necrotic material." He also describes in the spleen, "spherical nodules varying in size from 1 to 7 mm. in diameter" filled with odorless necrotic material. Similar nodules were found in the liver. A frequent lesion produced by another strain was an abscess of the orbit behind or below the eyeball. Cotton studied strains from Missouri and California and found the same type of lesion in all.

Weeter (24), in attempts to isolate the porcine strain from the uteri of swine, obtained cultures three times in 259 non-gravid uteri and once in 181 gravid uteri. Following the inoculation of guinea pigs, "two of the strains produced more lesions of the spleen than were given by many of the bovine strains, but these lesions were not more marked than were obtained with a freshly isolated culture from bovine sources."

Schroeder and Cotton (25) find that after placing 1 drop of a heavy suspension of *B. abortus* on the conjunctiva of guinea pigs there was 33 ⅓ per cent infection following the bovine type and 100 per cent following the porcine type. Placing 1 drop on tongue, 58 ⅓ per cent of porcine and none of bovine type infection resulted. Of twelve controls placed with these, none of bovine controls, but seven of porcine controls became infected. In these experiments it is not stated what constitutes infection.

Outbreaks of swine abortion appear to be restricted thus far to the Middle West and the western coast. Fresh cultures were, therefore, not obtainable for a renewed study of this type of *B. abortus*. In view of the practical importance of the subject, five strains received from outside sources were tested on guinea pigs. The meager but significant outcome may be briefly summarized.

Two strains were obtained in 1923, which had been isolated in

Missouri. These were tested on guinea pigs under the same conditions and at the same time that *B. melitensis* II and III were under observation. Four guinea pigs were inoculated from each strain. The animals were killed in the 3rd month following inoculation. In three animals inoculated from strain designated I, there were no lesions observable and cultures made with the entire spleen were negative. The fourth animal developed a bulging swelling on the inner aspect of the left knee joint which became centrally necrotic. Both thymus glands grew to twice the normal size and were filled with abscesses 4 to 5 mm. in diameter. The epididymis of right testicle was shrunken but not abscessed. *B. abortus* developed in all of three spleen cultures.

The four guinea pigs inoculated with the second strain were killed at the same time. None presented any enlarged spleen or diseased testicles, nor were lymph nodes appreciably swollen. From three, however, *B. abortus* was recovered from the spleen where it was present in very small numbers.

Recently the writer received, through the kindness of Dr. E. C. Schroeder, three strains of the porcine variety, which are designated below as III, IV, and V. III and V were isolated in Illinois, IV in Minnesota. Their total age in artificial media was not communicated. They grew luxuriantly in unsealed tubes. Four guinea pigs were inoculated with each strain. The dose used was slightly heavier than in preceding tests. 1 cc. of a $\frac{1}{500,000}$ dilution of the condensation water after washing the growth into it was injected subcutaneously in the left flank. The guinea pigs were killed between $2\frac{1}{2}$ and 3 months following inoculation.

The results indicated marked attenuation. In one guinea pig receiving Strain III, the left kneefold node contained a 5 mm. abscess. In the liver were eight to ten similar softened foci from 2 to 10 mm. in diameter. The spleen was small. In a second animal, yellow, soft foci occurred in the left kneefold nodes, in the right axillary, the submental, the cervical, and the left pelvic node. These various foci or abscesses were up to 4 mm. in diameter. The small spleen contained two 1 mm. foci. In the third animal, only the kneefold node nearest the site of inoculation had two softened foci. The fourth animal was negative. Spleen cultures remained sterile. The abscesses usually contained living bacilli.

In three of the guinea pigs receiving Strain IV, one or two small abscesses were found in the left kneefold nodes. The fourth animal was entirely negative. Cultures of spleen tissue were negative.

In one of the four guinea pigs receiving Strain V there were two small foci in the left kneefold node. In a second guinea pig, both left kneefold and one cervical node contained a centrally cheesy focus, 1.5 mm. in diameter. The two remaining animals were entirely normal. All spleen cultures remained sterile.

The results thus obtained from the five strains indicate a great decline in virulence if we take the published statements as a basis for our judgment.

<div align="center">DISCUSSION.</div>

The impression first made upon the writer, accustomed to the bovine disease, by the macroscopic appearance of the spleen, lymph nodes, and thymus of the guinea pigs inoculated with the human strains was that the disease was entirely distinct from that produced by *B. abortus*. This superficial impression was quickly reduced to proper dimensions by further study. The uniformity of the picture due to the bovine races contrasted with the quite different uniformity of lesions produced by the two human strains, isolated at different times in different sections of the country, furnishes strong evidence that the two types are not identical. The differences between them are obviously not great and in themselves supply evidence of the fundamental relationship of the bovine and the human strains. The question before us is not, however, the closeness of this relationship but the capacity of the bovine race to invade the human subject and multiply enough to produce symptoms characteristic of Malta fever.

The histological character of the focal lesions produced by the two races in guinea pigs may be briefly summed up as follows: Both produce in spleen and lymph nodes a proliferation of a certain type of cell which, for convenience, is called endothelial. It resembles the cells focalized by the tubercle bacillus without, however, undergoing the same retrogressive changes. The cells retain stains feebly and thus give the impression of rarefied areas under low powers. In the *B. abortus* animals the proliferation is not extensive. The groups

of cells are small, ranging from a few cells to masses 0.4 to 0.6 mm. in diameter. Frequently the groups coalesce into a broad meshwork throughout the organ. The amount of proliferation varies with the strain and age of the lesion. In very rare instances when the cell focus attains the maximum dimensions given, a nucleus of polymorphs may occupy the center. Necrosis has not been observed in any animal inoculated with early cultures. In only one case of many hundreds a small softened focus was found in the kneefold node of a guinea pig inoculated directly with uterine washings. In such cases it is rather remarkable that more cases do not occur in view of the rapid invasion of the postparturient uterus by cocci and bacilli of various kinds which are injected with *B. abortus* in the washings.

In the guinea pigs receiving the human strains the primary lesion is identical with that of the bovine disease. There is proliferation of the same cell type at the start. After the 2nd or 3rd week, some or all of the cell foci enlarge until they become visible to the naked eye as yellow masses 2 to 3 mm. in diameter, sharply outlined but not raised. Sections show extensive polymorph infiltration with or without central necrosis. When lymph nodes and thymus have enlarged to 1 cm. in diameter, it is probable that many foci have coalesced. The entire gland is then converted into a stiff mass enclosed in a thin-walled capsule.

The polymorph invasion and necrosis appear to run parallel with the size of the endothelial foci. Small foci remain unchanged. In the testicles the foci resulting from the two types ultimately suppurate. In the *B. abortus* animals this secondary change may be tentatively referred to the presence of masses of spermatozoa in the tubules which become imprisoned on account of the pressure of the interstitial foci. The compressed tubules have been observed to become centers of necrosis. In organs closed to the exterior, such as spleen and lymph nodes, polymorph invasion leading to softening has not been observed in *B. abortus* animals.

The injection of large numbers of *B. abortus* may lead locally to suppurative processes due to the presence of intracellular bacterial poisons. In the animals given minute doses, the early process is always an endothelial proliferation in lymph nodes and spleen. In-

jection into the peritoneal cavity and the subcutis produced the same type of disease, if we except a slight difference in the course of the lymph node lesions due to the original site of deposit of the bacteria. Following the intraperitoneal route, the testicles were somewhat earlier and more severely involved in both groups of guinea pigs.

The turn in the cellular reaction of guinea pigs infected with the human strains leading to a suppurative infiltration of the primary foci of endothelial elements suggested the idea that perhaps the blood picture of inoculated guinea pigs might reflect this turn and make a differential diagnosis between *B. abortus* and the human strains feasible. To gauge this possibility, Miss M. L. Orcutt studied the total and the differential count of the leucocytes of guinea pigs inoculated with the two strains of *B. abortus* (1114 and 1119) and the two strains of *B. melitensis*. To these were added two control animals. The counts were made once a week for 13 weeks. The results were disappointing in so far as they did not present differences wide enough for building on them any differential diagnosis between the *B. abortus*, the human, and the control guinea pigs.

To meet the possible objection that *B. abortus* may produce lesions similar to *B. melitensis* after it has been cultured for some time, six strains of *B. abortus* from five different States and originally isolated by the writer were injected into guinea pigs in doses corresponding to those used throughout in this study. The animals were killed after 2 and 3 months, respectively. In no case were lesions found undergoing suppurative changes. The decline in virulence resulted simply in a fading out of the lesions without a change in the character of the reaction. One strain of *B. abortus* isolated 6½ years ago had become avirulent. Two guinea pigs killed 63 and 83 days after inoculation, respectively, failed to show any lesions and cultures of the entire spleen were negative. The weight of the guinea pigs increased during this time from 405 and 410 gm. to 725 and 830 gm., respectively. Three strains from three different herds isolated 5 years ago still produced moderately enlarged spleens and localizations in one or both testicles. Spleen cultures were positive. One strain isolated 2 years, and another 15 months ago were less virulent than the preceding.

It is fairly obvious from the results obtained that strains which

have a long period of artificial culture behind them are not adapted for the determination of subtle differences, since the lesions produced tend to converge in a negative direction. This is emphasized by results obtained with a strain of *B. melitensis* isolated by Dr. Zammit from goat's milk on the Island of Malta in 1920. This strain, kindly sent by the Lister Institute, was inoculated into guinea pigs in the same doses used on *B. melitensis* II and III. Six guinea pigs were used. After inoculation the weights continued to increase. Two were killed after 27 days, two after 34 days, and two after 42 days. In none was there any spleen enlargement or other lesion. The entire spleens of four were cultured by tearing each into several bits and transferring to agar slants. In only one a growth appeared above the bit of spleen in the condensation water. This was identified as the organism inoculated.

Turning to the porcine race of *B. abortus* we find both in the published statements quoted and in the results of the guinea pig inoculations outlined above that this race resembles the two human strains much more closely than do the bovine strains. Whenever any lesions were produced they resembled those following inoculation with the human strains. We must, therefore, be prepared to look for the source of the human infections in the bacillus of swine abortion, provided the caprine type is not in evidence. The fact that Dr. Blake's patient was occupied in a slaughter house with handling fresh pork lends support to this view.[2]

When races resemble one another as closely as do the three strains of *B. abortus* there may be an occasional aberrant infection of one host with the strain from another host, as is true of the three races of tubercle bacilli. The close association of cattle and swine or of goats and cattle may lead to the infection of some abnormally susceptible member of one host species with the parasite of the other host. On the whole, however, such an event is probably rare.

A renewed thorough investigation of the effects of *B. melitensis* from the goat on guinea pigs is called for before the differential characters of the three primary races of this species can be clearly established. Slightly modified, intermediate types due to occasional

[2] Personal communication.

aberrant parasitism are to be anticipated. In the meantime, the evidence that *B. abortus* producing disease of the placenta in cattle may produce a disease in man simulating Malta fever must be regarded as inadequate in establishing any such relationship. The situation as it presents itself is very much like the one forced upon bacteriology in 1898 with reference to the tubercle bacillus. The problem is a thorough study of individual strains found in the human subject together with further investigations of the porcine strains.

A study of the agglutination affinities of the strains described in this paper was made by M. L. Orcutt (26). It will be seen by referring to her paper that with the technique used by her all strains acted alike.

CONCLUSIONS.

1. Comparative tests on guinea pigs with *B. abortus* yield the best results when minute doses are injected subcutaneously and the animal kept at least 4 weeks.

2. *B. abortus* gradually loses its virulence for guinea pigs under artificial cultivation.

3. Two cultures resembling *B. abortus* from cattle, isolated from human cases of so called Malta fever, are shown to be in their effect on guinea pigs not identical with the bovine strains.

4. The results of studies of *B. abortus* from swine indicate a close relation between the porcine strains and the two human strains.

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AGGLUTINATION AFFINITIES OF THE ABORTUS-MELITENSIS GROUP OF BACTERIA WITH SPECIAL REFERENCE TO TWO HUMAN STRAINS.

By MARION L. ORCUTT.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

In view of the differences between *B. abortus* of bovine origin and two human strains resembling *B. melitensis* described by T. Smith,[1] it seemed desirable to examine the serological relations of the strains compared by him with especial reference to agglutination.

According to Evans' first paper,[2] a *B. abortus* antiserum agglutinated both types of cultures alike, but a *melitensis* antiserum agglutinated *B. melitensis* to a higher titer than *B. abortus*. In absorption experiments there was little difference in the reactions with the *abortus* antiserum. The *melitensis* antiserum treated with *B. melitensis* lost all agglutinins for both cultures, but absorption with *B. abortus* removed only agglutinins for itself and left agglutinins for *B. melitensis* still present to a considerable degree.

Feusier and Meyer[3] made a serological study of fourteen strains. A series of agglutinin absorption tests resulted in the separation of the strains into four groups.

In 1923 Evans[4] reported on the serological classification of 49 strains from human, bovine, caprine, porcine, and equine sources, obtained from different parts of the world. These included strains from Groups 1, 2, and 3 of Meyer and Feusier. Altogether seven groups were created, three main groups and four smaller subgroups. More recently Evans[5] described eight serological groups. The only known method by which *B. abortus* and *B. melitensis* strains can be differentiated, according to Evans, is by the agglutinin absorption test, and by this method the differentiation is slight for saturation of a serum of either type with an antigen of the heterologous type will remove from 85 to 90 per cent of the

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[1] Smith, T., *J. Exp. Med.*, 1926, xliii, 207.

[2] Evans, A. C., *J. Infect. Dis.*, 1918, xxii, 580.

[3] Feusier, M. L., and Meyer, K. F., *J. Infect. Dis.*, 1920, xxvii, 185.

[4] Evans, A. C., *Pub. Health Rep.*, 1923, xxxviii, 1948.

[5] Evans, A. C., *Abstr. Bact.*, 1925, ix, 23.

agglutinins for the homologous antigen. Furthermore she found that the strains from Mediterranean countries showed a serological grouping distinct from strains from northern countries.

Zeller[6] obtained eight strains of *B. melitensis* from various institutes and compared them with strains of *B. abortus* which he had isolated himself. Sera obtained by immunizing rabbits with *B. abortus* and *B. melitensis* agglutinated both groups of cultures alike. He also found that after reciprocal absorptions of the sera by *B. abortus* and *B. melitensis* he could ascertain no differences between the two kinds of bacteria.

Skarić[7] worked with four *B. melitensis* strains and eight *B. abortus* cultures. Each type of serum agglutinated its own variety of culture to a higher titer than the other type and this was more marked with the *abortus* antiserum than with the *melitensis* antiserum. In the agglutinin absorption experiments only the homologous strain completely absorbed all agglutinins for all strains. The chief differentiation occurred by absorption of the *melitensis* antiserum with *B. abortus* cultures which completely removed agglutinins for *B. abortus* but only partially absorbed agglutinins for *B. melitensis*.

Khaled[8] made a comparative study of thirty strains of *B. abortus* and *B. melitensis*. He found that his *melitensis* antiserum agglutinated both sets of cultures alike, but the *abortus* antiserum agglutinated the *abortus* cultures to a slightly higher titer than the *melitensis* strains. His absorption results showed a distinction only when the *abortus* antiserum was absorbed by *B. melitensis*. This removed agglutinins for *B. melitensis* but not for *B. abortus*.

Burnet[9] states that a *melitensis* antiserum obtained either from a patient or from a goat or from treated rabbits or guinea pigs agglutinated *B. abortus* and *B. melitensis* cultures alike or *B. abortus* to a slightly higher titer. On the other hand, *B. abortus* antisera prepared from guinea pigs or rabbits always agglutinated *B. abortus* to a higher titer than *B. melitensis*. Thus he concluded that the *abortus* strains in general were composed of a more comprehensive antigen than *B. melitensis*. He also states that whatever the differences established by agglutination tests between *B. melitensis* and *B. abortus*, it is certain that one will find also well marked differences between authentic strains of *B. melitensis*; and if all the attempts at grouping have any significance, they only tend to establish relative distinctions between representatives of the same species.

Futamura[10] studied twenty strains of *B. abortus* isolated from cattle in Japan and compared these with two strains of *B. melitensis* and one strain of swine *B. abortus* from England. His results showed that antisera prepared from each type of culture gave an identical agglutination titer for each type of organism

[6] Zeller, H., *Berl. tierärztl. Woch.*, 1920, xxxvi, 345.

[7] Skarić, J., *Z. Hyg. u. Infectionskrankh.*, 1922, xcv, 358.

[8] Khaled, Z., *J. Hyg.*, 1921, xx, 319.

[9] Burnet, Et., *Arch. Inst. Pasteur Afrique Nord*, 1923, iii, 48.

[10] Futamura, H., *J. Jap. Soc. Vet. Sc.*, 1924, iii, 145.

when living cultures were used, but if the antigen was heated at 60°C. or 100°C. for an hour the *B. abortus* antiserum agglutinated *B. abortus* cultures more powerfully than *B. melitensis*. The results of absorption tests showed that the *abortus* and *melitensis* antisera contained 88 to 89 per cent of common agglutinins and 11 to 12 per cent of specific agglutinins. The common agglutinogens and those specific for *B. abortus* were thermostable and the specific *melitensis* agglutinogens were thermolabile. He found no serological difference between the bovine and swine strains.

Ficai and Alessandrini[11] found that a *melitensis* antiserum unheated agglutinated both *melitensis* and *abortus* cultures, but if the serum was heated at 60°C. it still agglutinated the *melitensis* culture but failed to agglutinate the *abortus* culture. On the other hand, an *abortus* antiserum unheated agglutinated both cultures, but heated at 65°C. it still agglutinated *B. abortus* cultures and failed to agglutinate *melitensis* cultures. A *melitensis* antiserum heated at 65°C. did not agglutinate either culture, while a *B. abortus* antiserum heated at this temperature still agglutinated *B. abortus* to the original titer and heated at 75°C. still gave a definite reaction.

In publications comparing *B. abortus* of bovine and swine origin there is agreement on their serological identity. Doyle and Spray[12] stated that the swine and bovine strains were alike serologically. Hayes[13] in some studies on swine abortion mentioned that absorption tests with sera from rabbits immunized against three hog strains and against two bovine strains and sera from guinea pigs immunized against one bovine strain and serum from a naturally infected cow showed no differences. Cotton[14] studied six strains of *B. abortus* obtained from abortion disease of swine occurring in various regions. All six of the swine strains were like the bovine strains serologically.

EXPERIMENTAL DATA.

The agglutination and agglutinin absorption experiments to be reported here were made with six strains designated *B. abortus* 1114, and *B. abortus* 1119 (of bovine origin); *B. melitensis* II (Blake), and *B. melitensis* III (Baltimore) (from human cases); and *B. abortus* Swine I, and *B. abortus* Swine II. For further data on these cultures the reader is referred to Smith's paper.[1]

Sera were prepared by immunizing rabbits with *B. abortus* 1119, *B. melitensis* II, *B. melitensis* III, and *B. abortus* Swine I cultures all heated at 60°C. for ½ hour.

[11] Ficai, G., and Alessandrini, A., *Ann. ig.*, 1925, xxxv, 1.

[12] Doyle, L. P., and Spray, R. S., *J. Infect. Dis.*, 1920, xxvii, 165.

[13] Hayes, F., *J. Am. Vet. Med. Assn.*, 1921–22, lx, N.S. xiii, 435.

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Complete series of agglutination and cross-agglutination tests were carried out and each serum was absorbed by all the cultures and then tested for agglutinins against each strain.   In general the method of Feusier and Meyer was followed in the absorption procedure.   The serum was diluted 1/5 or 1/10 with physiological salt solution.   The culture was grown on agar and suspended in salt solution.   Equal volumes of the diluted serum and culture suspension were mixed and incubated at 37°C. for 2 hours and then refrigerated overnight.   The mixture was centrifuged the next day and the clear serum tested with the absorbing strain for complete absorption.   If agglutinins still remained for the absorbing strain, then more culture was added to complete the absorption before testing the serum against the other strains.

The results showed that each serum agglutinated all the strains equally, and after absorption according to the above method each strain removed all agglutinins for the homologous strain as well as for the other strains.   The tabulated results of a complete agglutination and absorption experiment with one serum will illustrate the reactions obtained in all cases.

All the cultures absorbed all agglutinins leaving none for homologous or heterologous strains.   This was the case with each lot of serum. These results indicate that these strains cannot be separated or distinguished by agglutination and absorption tests carried out as described above.   B. abortus 1114 and B. abortus 1119 are, however, known to be bovine strains.   The fact that the two swine strains, S.I and S.II, acted like the bovine cultures is in agreement with other workers who state that bovine and swine strains of B. abortus are alike serologically.   Since the strains B. melitensis II and B. melitensis III were identical in their serological behavior with B. abortus 1119 and B. abortus 1114 they may be classed as the bovine type serologically.

In making experiments with heated sera according to the methods of Ficai and Alessandrini we found that the B. abortus 1119 antiserum after having been heated at 65°C. for ½ hour still agglutinated all the cultures to the original titer.   Therefore, according to Ficai and Alessandrini, the B. melitensis II and B. melitensis III strains behaved like bovine type cultures in heated abortus antiserum. The heating experiments were made also with B. melitensis II and B. melitensis III antisera.   Here again after heating at 60°C. for ½ hour these sera still agglutinated both the B. abortus and B.

TABLE I.

B. abortus 1119 (Bovine) Antiserum.

Agglutination titer with homologous strain (B. ab. 1119).

	Agglutinated by	1:40	1:80	1:160	1:320	1:640	1:1,280
Before absorption.	B. ab. 1119	C.	C.	C.	C.	++++	+
After absorption by							
B. ab. 1119.........	"	—	—	—	—	—	—
" 1114.........	"	—	—	—	—	—	—
B. melit. II.........	"	—	—	—	—	—	—
" III.........	"	—	—	—	—	—	—
B. ab. S. I..........	"	—	—	—	—	—	—
" " II.........	"	—	—	—	—	—	—

Agglutination titer with non-homologous strains.

	Agglutinated by	1:40	1:80	1:160	1:320	1:640	1:1,280
Before absorption.	B. ab. 1114	C.	C.	C.	C.	+++	+
	B. melit. II	C.	C.	C.	C.	++	+
	" III	C.	C.	C.	C.	+++	+
	B. ab. S. I	C.	C.	++++	++++	+++	++
	" " II	C.	C.	++++	++++	++	+
After absorption by							
B. ab. 1119.........	B. ab. 1114	—	—	—	—	—	—
" 1114.........	"	—	—	—	—	—	—
B. melit. II.........	"	—	—	—	—	—	—
" III.........	"	—	—	—	—	—	—
B. ab. S. I..........	"	—	—	—	—	—	—
" " II.........	"	—	—	—	—	—	—
B. ab. 1119.........	B. melit. II	—	—	—	—	—	—
" 1114.........	"	—	—	—	—	—	—
B. melit. II.........	"	—	—	—	—	—	—
" III.........	"	—	—	—	—	—	—
B. ab. S. I..........	"	—	—	—	—	—	—
" " II.........	"	—	—	—	—	—	—
B. ab. 1119.........	B. melit. III	—	—	—	—	—	—
" 1114.........	"	—	—	—	—	—	—
B. melit. II.........	"	—	—	—	—	—	—
" III.........	"	—	—	—	—	—	—
B. ab. S. I..........	"	—	—	—	—	—	—
" " II.........	"	—	—	—	—	—	—-

TABLE I—*Concluded.*

After absorption by	Agglutinated by	Dilutions.					
		1:40	1:80	1:160	1:320	1:640	1:1,280
B. ab. 1119.........	B. ab. S. I	—	—	—	—	—	—
" 1114.........	"	—	—	—	—	—	—
B. melit. II.........	"	—	—	—	—	—	—
" III.........	"	—	—	—	—	—	—
B. ab. S. I.........	"	—	—	—	—	—	—
" " II.........	"	—	—	—	—	—	—
B. ab. 1119.........	B. ab. S. II	—	—	—	—	—	—
" 1114.........	"	—	—	—	—,	—	—
B. melit. II.........	"	—	—	—	—	—	—
" III.........	"	—	—	—	—	—	—
B. ab. S. I.........	"	—	—	—	—	—	—
" " II.........	"	—	—	—	—	—	—

melitensis cultures to the original titer, but in the lower dilutions of the heated *B. melitensis* III antiserum a zone of inhibition occurred for all cultures. Since the *B. abortus* strains (1114 and 1119) were agglutinated to the original titer in the heated *B. melitensis* II and *B. melitensis* III antisera, then, according to Ficai and Alessandrini, these sera behaved like *B. abortus* antisera. In the previous test it was seen that strains *B. melitensis* II and III behaved like *abortus* cultures in the inactivated *abortus* antiserum. Therefore, if Ficai and Alessandrini's method of distinguishing *B. abortus* and *B. melitensis* agglutinins by heat is correct, these results are further evidence for placing strains *B. melitensis* II and III serologically with the *abortus* type of organism. Heating *B. abortus* Swine I antiserum gave results corresponding to the bovine type, but a marked zone of inhibition for all cultures occurred in the lower dilutions. Since no differences occurred at the temperatures of 60°C. and 65°C., found critical by Ficai and Alessandrini, a higher temperature of 75°C. was tried. *B. abortus* 1119, *B. melitensis* II and III, and *B. abortus* Swine I antisera heated at 75°C. for ½ hour gave no agglutination with any of the six cultures. Thus in all experiments with heated sera all the strains behaved alike.

TABLE II.

B. melitensis II Antiserum.

Culture.	Unheated.						Heated (60°C.—1/2 hr.).					
	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:80	1:160	1:320	1:640	1:1,280	1:2,560
B. melit. II unheated	C.	C.	C.	C.	+++	+++	C.	C.	+++	C.	C.	++
" II heated 100°C.—2 hrs.	C.	C.	C.	C.	+++	+++	C.	C.	+++	+++	++	++
B. ab. 1119 unheated	C.	C.	C.	C.	+++	+++	C.	C.	C.	C.	C.	+
" 1119 heated 100°C.—2 hrs.	C.	C.	C.	C.	+++	+++	+++	+++	+++	+++	++	+

B. abortus 1119 Antiserum.

Culture.	Unheated.						Heated (65°C.—1/2 hr.).					
	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:80	1:160	1:320	1:640	1:1,280	1:2,560
B. melit. II unheated	C.	C.	C.	++	±	–	+++	+++	C.	C.	++	+
" II heated 100°C.—2 hrs.	C.	C.	+++	++	+	–	+++	+++	+++	++	+	+
B. ab. 1119 unheated	C.	C.	C.	++++	+++	–	+++	+++	C.	C.	+++	+
" 1119 heated 100°C.—2 hrs.	C.	C.	++++	++	+	–	+++	++	+++	+++	++	+

Futamura used heated antigens and claimed a difference in reaction in the *abortus* antiserum. He also stated that specific *melitensis* antigen was heat-labile and specific *abortus* antigen and the common antigen were heat-stable. This would mean that a *melitensis* culture after having been heated would give a lower agglutination titer, and if such a culture was combined with a heated *melitensis* serum which contained only specific *melitensis* agglutinins (Ficai and Alessandrini), then the titer would be negative. With these points in mind some experiments were made with heated and unheated cultures in heated and unheated sera. The results with two strains are given in Table II.

The results showed that the agglutinins and agglutinogens of all the cultures were heat-stable at the given temperatures. There was no evidence of a heat-labile specific *melitensis* antigen in the strains *B. melitensis* II and *B. melitensis* III.

CONCLUSIONS.

The conclusions to be drawn from this study are that while there appears to be evidence of serological distinctions between *B. abortus* and *B. melitensis* cultures as studied by earlier workers, the experiments reported in this paper show no serological distinctions among the six strains used in the study. *B. melitensis* II and *B. melitensis* III were found to be identical with the bovine and swine strains by means of agglutination and agglutinin absorption reactions in unheated sera, by agglutination in heated sera, and with the use of heated cultures in unheated and heated sera.

ALLERGIC IRRITABILITY.

III. The Influence of Chronic Infections and of Trypan Blue on the Formation of Specific Antibodies.

By PAUL A. LEWIS, M.D., and DOROTHY LOOMIS.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

The purpose of this paper is to present observations in confirmation and extension of our previous papers (1, 2) on this general subject, particularly the first paper. We there showed that in the tuberculous guinea pig the production of anti-sheep hemolytic amboceptor was exaggerated. We have now found that the production of agglutinins for *Bacillus typhosus* is likewise increased by preexisting active tuberculosis. We have studied other mild infections, the influence of the dead tubercle bacillus, and trypan blue, finding that hemolytic amboceptor production may be increased by these means. We likewise have been able to increase measurably the production of hemolytic amboceptor by the rabbit through the establishment of an infection with *Bacillus tuberculosis*. These additional observations seem to warrant the conclusion that the phenomenon dealt with is a very general one, as to the species in which it can be made evident, the antigens whose activity may be influenced, and the means which may be effective in altering the animal's reaction capacity. These additional observations and others which will appear incidentally in the body of the paper suggest a further definition of the conception of allergic irritability as we have sought to develop it.

As an incident to our previous observations on increased antibody production we determined the curve of antibody production in the guinea pig and found that it departed from the usual type. The present paper contains some additional information on this point.

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When preparing our previous paper we overlooked a paper by Clark, Zellmer, and Stone (3). These authors found that the production of agglutinins for *B. typhosus* was increased by the previous administration of heat-killed Gram-positive cocci. Rabbits were used. Some evidence that the rabbits were measurably protected against killing doses of *B. typhosus* was also obtained by these authors. These experiments are of importance as a possible indication of some connection between the experiments on antibody production and the classical experiments of Pfeiffer and Issaeff (4) on immunity induced by the injection of bouillon and other indifferent substances into the peritoneal cavity. It has usually been assumed that the effect obtained by Pfeiffer and Issaeff was of short duration and purely a local manifestation. It may possibly be related in some way to a more general and lasting reaction.

Khanolkar (5) obtained a moderate increase in the production of agglutinins for *B. paratyphosus*, Gärtner, by previous treatment with killed *B. pyocyaneus*. He failed to obtain such an effect with staphylococci and ricin.

Schroeder (6) has observed greatly increased anti-sheep amboceptor production in rabbits which developed abscesses at the site of inoculation of erythrocytes. In control experiments she was successful in showing the similar influence of pneumococcus infection. With staphylococci and *B. pyogenes* she did not succeed in establishing chronic infections and was unable to show any stimulating influence.

Hektoen and Corper (7) have recently reported that they failed to obtain increased antibody production in rabbits under the influence of preexisting tuberculosis infection with any degree of consistency. Their results were not entirely negative, however, even as reflected in their statement of conclusions, and we feel that they are to be interpreted as in essential harmony with our own. This matter will be taken up in greater detail in our discussion.

Gay and Clark (8) found that administration of trypan blue interfered with the production of antibodies. This is interpreted as probably signifying that those cells in the body which have a marked affinity for trypan blue are concerned in a vital way in the production of antibodies, and that this function is interfered with when they are filled with dye. The authors' context implies that the "blocking" experiments of others have sometimes given increased antibody production rather than the anticipated decrease. There is probably no fundamental contradiction between the opposing results, each tending to assign to the reticulo-endothelial cell system a definite place in antibody production.

In a first series guinea pigs belonging to our inbred Family 13 were used. They were given at one time 5 cc. subcutaneously and 5 cc. intraperitoneally of a 20 per cent suspension of washed sheep red blood corpuscles. They were further treated as follows: A group of five received 5 cc. of 1 per cent trypan blue intraperitoneally the day before the sheep blood, and 2 cc. each day thereafter until

the end of the experiment.   A second group of five received 0.8 cc. of a 24 hour culture of a streptococcus subcutaneously 2 days before the injection of sheep cells.
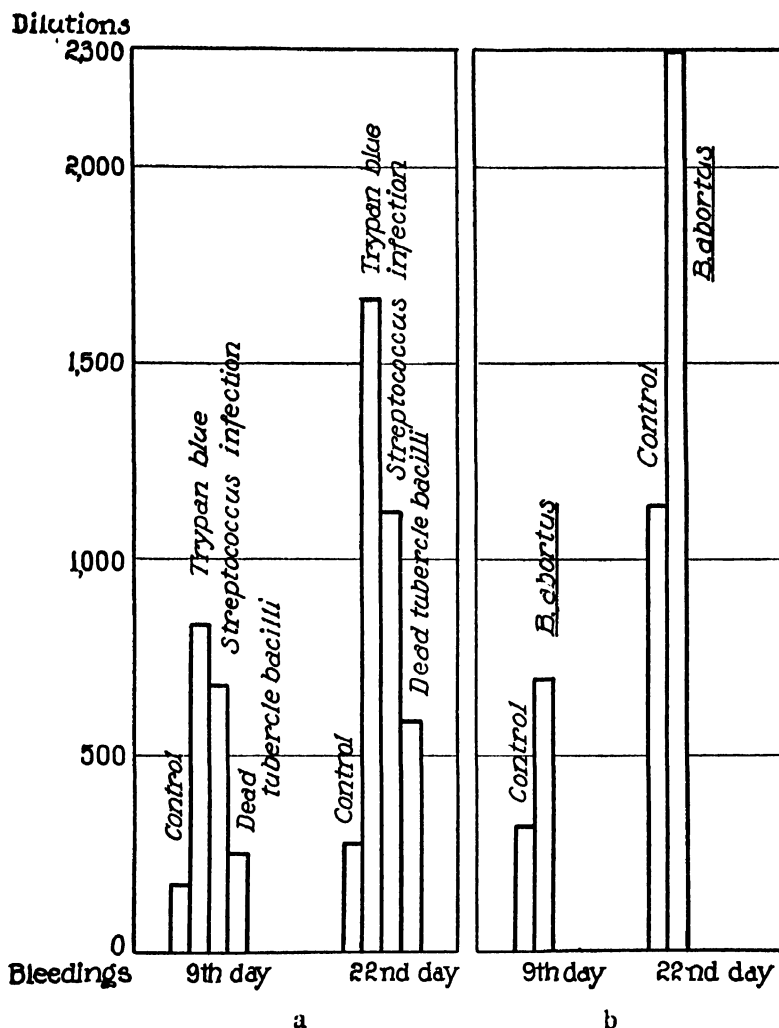
CHART 1, *a* and *b*.   (*a*) Average hemolytic amboceptor production of groups of guinea pigs treated with trypan blue, a streptococcus, and dead tubercle bacilli contrasted with a control group.   Bleedings on the 9th and 22nd days after the administration of sheep erythrocytes.   (*b*) Same for a group infected with *B. abortus* contrasted with a control group.

The streptococcus was one isolated some months earlier from a swollen lymph node in a guinea pig dead of some cause unknown.   A third group of five received 2 mg.

of culture of *B. tuberculosis*, Bovine xiv, intraperitoneally 2 days before the sheep cells. The culture was killed by heating to 60°C. for 1 hour. A fourth group of five was used for control, receiving no treatment other than the sheep red cells.

The animals were bled on the 9th and 22nd days following the erythrocyte injections. The sera were tested individually for the minimum hemolytic dose against 0.1 cc. of a 10 per cent suspension of sheep erythrocytes, in the presence of an excess of complement and in a total volume of 1.1 cc. The temperature of the test was 35–36°C., the time 1 hour, and the readings were made at once. For purposes of interpretation the results were averaged for each group. As a check the sera for each group were pooled in equal portions and the combined serum tested at a later time in the same way. The results were in essential accord with the averages of the individual determinations.

The results of this series of tests are shown in Chart 1, *a*.

In a second series ten animals of inbred Family 13 and ten of Family 35 were inoculated with 1 cc. of a suspension of *B. abortus* grown on agar and suspended in normal saline solution to a density of 2.5 on the Gates gauge. 21 days later these animals with five others of each family, selected as controls, were treated with sheep red cells. They were given at one time 5 cc. intraperitoneally and 5 cc. subcutaneously as in the earlier series. These animals were likewise bled on the 9th and 22nd days after the blood injection and the hemolytic titer tested as before.

The results of this series are shown in Chart 1, *b*.

Two experiments were next carried out ·to see if infection with *Bacillus tuberculosis* would affect the production of antibodies other than sheep cell hemolysin. In the first experiment the first treatment with *Bacillus typhosus* proved insufficient for agglutinin production. A second treatment was therefore given. The tuberculous animals gave much more agglutinin than the normals, but since we have wished for a result uncomplicated by repeated injections with the test antigen, we carried out a second series as follows:

Twenty guinea pigs of inbred Family 13 were given an intraperitoneal injection of 1/100 mg. of *B. tuberculosis*, Bovine xiv. 2 weeks later these animals with ten others used as normal controls were given at the one time 1 cc. intraperitoneally and 1 cc. subcutaneously of a suspension of *B. typhosus*, density with Gates gauge 1.8, which had been heated to 55°C. for 1 hour. All the animals were bled on the 7th, 9th, 11th, 15th, 18th, 20th, and 22nd days after the injection of *B. typhosus*. Equal parts of the serum of each animal were pooled by groups for each day, stored in the ice box, and the whole tested with the same suspension of

B. typhosus that had been used for the injection. The agglutination test was done by the macroscopic method. The tubes were incubated for ½ hour at 56°C., placed in the refrigerator overnight, and read the following morning. The last tube showing complete clearing with heavy flocculi on reshaking was taken as the end-point.

The result of the whole experiment is shown in Chart 2.

Having thus found that a stimulus to antibody production could be attained in several ways, and that the resultant increase could be
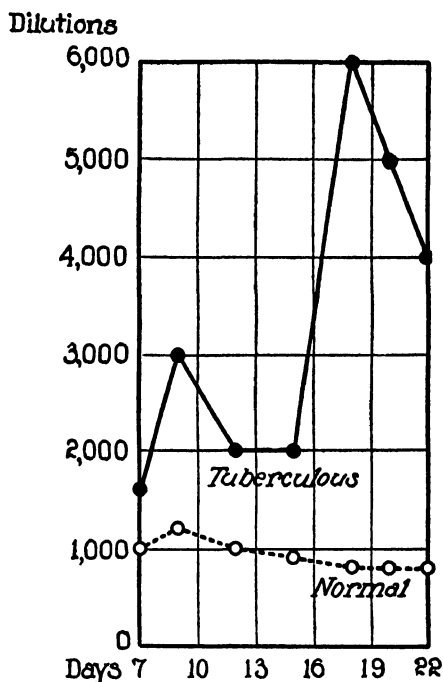
CHART 2. *B. typhosus* agglutinin production in tuberculous and normal animals. The time in days is counted from the administration of *B. typhosus* as described in the text.

observed with a bacterial antibody as well as with an hemolytic amboceptor, we tried to see if the effect could be obtained in an animal other than the guinea pig. We chose to produce anti-sheep hemolytic amboceptor in the rabbit under the modifying influence of an infection with *Bacillus tuberculosis*. The experiment was planned to fulfill conditions as they had been developed in the guinea pig;

that is, it appeared that the infection must be of at least 2 weeks standing if the effect was to be manifest. Three experiments were done.

In the first of these 1/100 mg. of a bovine type culture, No. xviii, was given intraperitoneally. No significant differences in hemolysin production were made out, and when the animals were autopsied it was found that the infection had produced an insignificant amount of disease. In a second experiment 1 mg. of bovine type culture No. xiv was given intravenously. The blood cells were injected on the 16th day. On the 4th, 7th, and 9th days after the erythrocyte injection the amboceptor content of the tuberculous rabbits was lower than in the controls. Thereafter there was but one surviving tuberculous rabbit. As the amboceptor content of the control animals remained stationary over the succeeding 6 days, that in the tuberculous animal increased until on the 16th day it stood at 1/16,000. The average of the controls on this day was 1/5,600 and the maximum among five was 1/9,000.

TABLE I.

Controls.			Tuberculous.		
Rabbit No.	7th day.	18th day.	Rabbit No.	7th day.	18th day.
31	1/2,000	1/1,600	24	1/10,000	Dead.
32	1/4,000	1/5,000	25	1/3,000	"
33	1/5,000	1/3,000	26	1/8,000	"
34	1/4,000	1/1,600	27	1/20,000	1/12,000
35	1/3,000	1/1,600	28	1/8,000	Dead.
			30	1/8,000	1/12,000
Average....	1/3,600	1/2,540		1/9,500	1/12,000

In a third experiment the same culture was used (Bovine xiv). 1/20 mg. was injected intravenously into seven rabbits. 17 days later these together with five controls were given at one time 5 cc. of 20 per cent washed sheep red blood corpuscles intravenously, 5 cc. intraperitoneally, and 5 cc. subcutaneously. They were all bled on the 7th and 18th days succeeding. The results are shown in Table I.

While it is plain that the experiment does not proceed with the same regularity as in the guinea pig, it is nonetheless evident that in the rabbit, under appropriate conditions, an infection with the tubercle bacillus does increase the production of anti-sheep amboceptor. Conditions favorable to the demonstration appear to be the thorough establishment of an infection of considerable, but not of overwhelming severity.

The preceding experiments show that the stimulation of antibody production with which we are concerned is quite a general phenomenon. It can be brought about by a variety of chronic infections; it is exerted against at least two antigens, and is developed in at least two species of animals. Of the means adopted none thus far tested is equal from a quantitative point of view to an active, well established infection with *Bacillus tuberculosis*.

In this connection it has been interesting to compare the effect of repeated injections of red cells in normal and tuberculous animals. The experiment is limited in its scope because of the short length of life of the tuberculous. The results may be simply stated without tabulation. In a group of ten tuberculous animals given three injections of red cells an average amboceptor titration of 1/58,000 was obtained on the 22nd day after the last injection. One of the animals reached 1/100,000. In normal animals the highest individual figure obtained by any number of injections (following the second injection in this instance) was 1/5,500, and the highest average for any group was 1/4,500. As reported in our first paper, a single injection of blood cells in tuberculous animals may give an average titration of over 1/20,000 on the 22nd day, a figure still four or five times the maximum we have been able to attain by repeated blood cell injection in uninfected animals. The stimulus of infection would appear to be more effective in increasing the quantity of antibody produced than the stimulus of several preceding injections of the specific antigen. That the figures for attainable antibody concentration afford the proper basis for comparison of the quantitative effects of the two sorts of stimuli may well be doubted. We have wished to construct complete curves of antibody production for the normal and the tuberculous after repeated blood cell injections. The technical difficulties have not so far been surmounted. It is our impression, however, that for repeated injections, as was shown for single injections in our first paper, the form of the curves would be comparable but at a higher level. It seems likely, in other words, that the factor by which each succeeding injection would increase the results of its predecessor is no greater in the case of the tuberculous animal than in the normal. From this point of view the two kinds of stimuli seem to act quite independently and to be capable of summation.

As an incident to our observations as previously reported, we determined the curve of antibody production in the guinea pig for anti-sheep hemolytic amboceptor. We found it characterized by a double maximum, the first and usual peak at about the 9th day being succeeded by a low point at about the 12th day and a later peak, higher than the first, about the 22nd day. In the course of the present work we have amplified these observations to a certain extent. As shown in Chart 2, the curve as described is followed in its characteristic features as a response to a single injection of *Bacillus typhosus* (killed culture). In this case the second maximum appears somewhat earlier, at the 18th rather than the 22nd day.
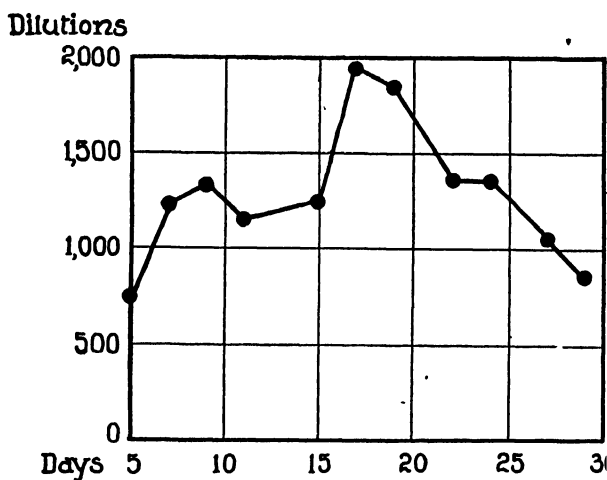
CHART 3. Composite curve of hemolytic amboceptor production in guinea pigs after the fourth of a series of treatments with sheep cells at 28 day intervals.

In such work as we have done with rabbits we have kept this question constantly in mind but have obtained no suggestion of a second peak in them.

In Chart 3 we present the results of observations on a group of guinea pigs for the period succeeding the fourth of a series of injections of sheep red cells at 28 day intervals. It will be noticed that the form of the curve is essentially that of Chart 2 in this paper and those previously published. The first peak of production occurs unchanged at the 9th day. The second peak is advanced to the 17th to 19th day.

As was to be expected, observations made after the last of a series of injections at 5 to 7 day intervals do not develop this form. The high point is usually about the 9th day in such cases, probably because this day following the last injection is also near the second peak of some previous one.

<div align="center">DISCUSSION.</div>

In our previous papers we have defined and used the term "allergic irritability" as a "general characteristic of the animal on the basis of which it reacts to stimuli of the antigenic class, whether they be helpful, injurious, or indifferent to bodily health." The observations herein recorded serve to confirm and amplify the evidence that such a distinction between the actual response of the animal and its "capacity" to respond is of interest. The allergic irritability is found to be increased by a variety of means, particularly by several chronic infections. Infection with the tubercle bacillus under conditions favorable to the full development of its influence is the most effective of these so far as our experience goes.

An intensive treatment with trypan blue throughout the course of the reaction period was almost as effective as infection with the tubercle bacillus. This substance was chosen for experiment because of its well known affinity as a vital stain for cells of the reticulo-endothelial cell system particularly the macrophages. There is considerable evidence for the assumption that these cells are increased in number by treatment with trypan blue and related substances. It is likewise to be accepted that cells of this system are greatly proliferated in tuberculosis. Correlation of these suggestions with such evidence as is available as to the origin of antibodies in these cells, and this is also considerable, lends weight to the thought that the stimulation of antibody production which we have observed is based on an increased activity of these cells, due to either an increase in their number or a stimulation of one or more phases of their physiological activity.

There seems to us to be no essential conflict between this conception, or the concrete experiments on which it is based, and the work of others who have succeeded in inhibiting antibody formation by "blocking" these cells with trypan blue and other colloidal substances.

It is, in fact, a rather general rule that substances which exert physiological activity are stimulating in certain doses and depressing when their application is carried to an extreme.

In any event our experiments justify a more serious consideration of allergic irritability as a characteristic, subject to experimental influence in connection with the general principles and phenomena of immunity. It is evidently possible while leaving the specific reactions to antigenic substances intact, to increase greatly their intensity by influences that affect a more general,—one is tempted to say "non-specific,"—set of reactions.

CONCLUSIONS.

1. The allergic irritability of the guinea pig (capacity of the animal to react to antigenic substances) is increased by infection with *Bacillus abortus* and a streptococcus, by the dead tubercle bacillus, and by intensive treatment with trypan blue, respectively. The effect of these influences, while definite, is less pronounced than that previously found for infection with the tubercle bacillus. The production of anti-sheep hemolytic amboceptor was used as the test reaction in these cases.

2. The allergic irritability of the guinea pig with reference to antityphoid agglutinin is increased by infection with the tubercle bacillus.

3. The allergic irritability of the rabbit with reference to anti-sheep hemolytic amboceptor is increased by an infection of suitable severity with the tubercle bacillus.

4. In the guinea pig the curve of antibody production is complex. Its peculiarities are developed during the production of antityphoid agglutinins as well as that of anti-sheep hemolytic amboceptor. In the latter case injections of antigen subsequent to the first give rise to a curve of production unchanged in form but somewhat affected in the time relations.

5. The effects of infection with *Bacillus tuberculosis* on allergic irritability with reference to anti-sheep hemolytic amboceptor are operative throughout a course of immunizing treatments. The successive increases due to the cumulative effect of repeated doses of the antigen are developed on a higher level. The end-result is that the animal with increased irritability furnishes more antibody not

only in response to the initial injection of antigen as previously described, but an absolute increase over the amount attainable by a comparable number of treatments in series. That portion of the final result contributed by the increase in allergic irritability appears to be no less, and may even in instances be somewhat more than that due to the earlier doses of the specific antigen.

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VARIATIONS IN CO₂ REQUIREMENTS AMONG BOVINE STRAINS OF BACILLUS ABORTUS.

By THEOBALD SMITH, M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

In a former publication,[1] the importance of CO_2 above a certain minimum concentration in stimulating the multiplication of freshly isolated strains of *Bacillus abortus* was pointed out and the literature cited. CO_2 was shown to be necessary not merely as an adjuster of the hydrogen ion concentration but possibly as a source of carbon. In estimating the significance of this phenomenon it is necessary to keep in mind certain other characters of *Bacillus abortus*. This organism is aerobic, for without oxygen it fails to multiply. A continuing supply even of small quantities is sufficient. Growth in sealed tubes is always less abundant than in unsealed tubes after the strain has become even slightly saprophytized. On the other hand, the freshly isolated strain, which will be called the parasitic strain, grows poorly in air and soon dies out unless large numbers of living bacilli are deposited on the agar surface. Even then the growth remains focal. This localized multiplication was regarded as indicating a community effort in the production of CO_2 to favor the multiplication of at least one or a few of the bacilli deposited on the new substrate. Frequently the focal growths under the condition described, *i.e.* in open tubes to which large masses of bacilli have been transferred, appear first on the lateral margins of the slope as if some influence of the glass container was favorable. When tubes of NaOH were hung below the plug and the tubes sealed, growth frequently appeared first, if at all, between the agar and the glass. It is probably some association with a minute source and a reservoir of CO_2 that is here involved and this may

[1] Smith, T., *J. Exp. Med.*, 1924, xl, 219.

account for the original observation of Bang and Stribolt of two optima of growth in their deep, semisolid medium. They, however, refer them to two optima of O-tension.

<h3 style="text-align:center">EXPERIMENTAL.</h3>

The existence of strains departing more or less in their CO_2 relations from the more common type had already been demonstrated[1] and the next step to be taken was to determine the relative frequency of such strains and their possible relation to cultures used as vaccines. The strains which fail to grow in open tubes even in the presence of abundant seed and which fail in sealed tubes in high dilutions are the most common and widely distributed and may be considered the normal or type bovine strain.

The registering of differences toward CO_2 did not appear feasible since all strains grow well and even luxuriantly in the presence of small amounts of CO_2. It was therefore determined to use different dilutions of seed in tubes of nutrient agar hermetically sealed to measure the sensitiveness of freshly isolated strains to CO_2 as it accumulates to a given threshold value in the sealed tube. This method was described in the first paper. It remained to choose some definite series of dilutions and to standardize the method as far as possible without making it cumbersome. After various trials the following scheme was chosen for the routine testing of all freshly isolated cultures in 1924 and 1925.

Ordinary veal-infusion agar in quantities of 6 cc. sloped in test-tubes of ⅜ to ½ inch diameter was used for the initial culture. The condensation water was about ⅓ cc. in volume and 1 cm. deep. After the original culture from fetus or inoculated guinea pig had reached its maximum growth a mass of bacilli was transferred with loop or wire and rubbed over the entire surface. In such a sub-culture properly sealed a filmy continuous growth appears within 2 or 3 days. This film is as a rule of nearly equal vigor in all sealed cultures, the seal favoring the parasitic and restraining the saprophytic types. The growth at the end of 2 to 3 days is washed down with the condensation water. This becomes heavily turbid. A few drops of bouillon should be added if the condensation water is not of the requisite depth.

This suspension is used to prepare dilutions. The writer had adopted the arbitrary scheme given below as requiring but little glassware. Two pipettes supplied with rubber "teats" or bulbs were drawn out so as to deliver 25 drops

ʲᵉr cc. A platinum loop was made of 24 gauge wire and 5 mm. external diameter ᵻnd was found to deliver about 1/250 cc. of the suspension. The dilutions were ₍made in 3 cc. bouillon:

(a) 1 loop of condensation water suspension to agar (control).
(b) $\frac{2}{25}$ cc. (2 drops) " " " " 3 cc. bouillon ($\frac{2}{25}$ dilution).
(c) $\frac{2}{250}$ cc. (2 loops) condensation water suspension to 3 cc. bouillon ($\frac{1}{875}$ dilution).
(d) $\frac{1}{25}$ cc. (1 drop) of (b) to 3 cc. bouillon ($\frac{1}{2,800}$ approximate dilution).
(e) $\frac{2}{250}$ " (2 loops) " (b) " 3 " " ($\frac{1}{14,000}$ " ").
(f) $\frac{3}{250}$ " (3 ") " (c) " 3 " " ($\frac{1}{140,000}$ " ").
(g) $\frac{2}{250}$ " (2 ") " (d) " 3 " " ($\frac{1}{1,054,000}$ " ").

One standard loop of each suspension in bouillon is transferred to sloped agar.

Two series may be prepared, one of unsealed and one of sealed tubes. Whoever undertakes this work will soon discover how far to go with these two series. Thus with the normal type of *B. abortus*, the growth in (a) unsealed is restricted to a few centers on the agar slope, or may appear only in the condensation water; (b) unsealed fails to develop. In the series of sealed tubes, (a) always develops, (d) rarely grows, and some strains fail to show any growth in (c). On the other hand, strains which have been under cultivation 2 or 3 years may multiply promptly not only in all sealed tubes but in all open tubes of the series and even several steps beyond the scheme as far as given. In the open series the cotton plugs must not be dipped in paraffin as this may act as a partial seal and modify the result.[2] The method as given may be placed on a decimal system of dilutions by using pipettes graduated for the purpose. It is important, however, to bear in mind that extreme accuracy and uniformity are not attainable in a biological test of this kind without an expenditure of time scarcely justified by the results to be obtained.

The sealing requires some care to eliminate small gaps and avoid cracks later on. Irregularities in the dilution series may be due to them. It has been customary to place a circular disc of thin asbestos on the cotton plug as a base and drop the

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[2] In earlier work[3] it is stated that some strains grew in the second transfer without *B. subtilis*. This is probably to be accounted for by the procedure used in the writer's laboratory of dipping all plugs of agar tubes into paraffin to prevent too rapid evaporation. Sometimes the dipping was overdone and practically sealed the tube.

[3] Smith, T., and Fabyan, M., *Centr. Bakt., 1. Abt., Orig.,* 1912, lxi, 549.

sealing wax, softened in the flame, on it.   A thin iron spatula heated in the flame is useful in smoothing the surface, filling gaps, and removing any laterally projecting edges which later on may be knocked off when tubes are handled.   This accident may break the seal.   The plug of sealing wax is removed by piercing it with a heated iron wire curved and pointed at one end.   The tube is then heated in the flame to loosen the seal and the plug lifted out with the wire inserted in the hole.   The layer of wax need not be thicker than 1 mm. if properly manipulated.

It has been assumed that the sealing tends to bring the CO$_2$ concentration to a given level which permits multiplication into visible colonies or films.   The present paper brings no direct proof of this assumption.   An indirect proof was brought by the following simple experiment.

Three agar slants inoculated with dilution (*b*) of a very slowly growing strain were prepared.   One was left unsealed and the second was sealed.   The third was connected by means of a curved glass tube and rubber connections to a 24 hour growth of a saprophytized strain of *B. abortus*.   Within 2 days there was active multiplication in the third tube and in 3 days a very vigorous film.   The sealed culture was free from visible growth on the 15th day.   The unsealed tube remained as was anticipated permanently free from growth.   In brief, the saprophytized strain was producing enough CO$_2$ to assist the parasitic strain to a vigorous multiplication besides supplying its own needs.

Results of the Sealing of Graded Dilutions.—The following data, unless otherwise indicated, were obtained from the first, or less commonly the second transfer from the original culture from fetus or inoculated guinea pig.   It had already been stated that passage through a guinea pig does not modify appreciably the CO$_2$ relation. A number of determinations of strains isolated both from the fetus directly and from guinea pigs 1 to 3 months later are the basis for this statement.   The differences encountered in the strains are best brought out by two illustrations.

Fetus 1175.—*Culture from Fetus* (lung).
In the open series: (*a*) One colony in 13 days.
In the sealed series: (*a*) Growth in 2 days; (*b*) in 8 days.
                    (*c*) and (*d*) No growth in 19 days.
                Culture from Inoculated Guinea Pig.
In the open series: (*a*) One colony in 4 days.
In the sealed series: (*a*) Growth in 4 days; (*b*) in 5 days.
                    (*c*) No growth in 11 days.

Fetus 1187.—Culture from Fetus (fourth stomach).
Unsealed series; (*a*) Complete film in 2 days; (*b*) in 2 days.
Sealed series: (*a*) Growth in 2 days; (*b*) in 3–5 days; (*c*) in 2 days; (*d*) in 2–3
  days.

To illustrate further the two types of growth, the data of 1924–25 have been brought together in Table I.

It will not be difficult to select the more saprophytic strains from this table. They are 736, 916, 1119, 1128, 1176, 1186, 1187, 1207, 1211, and 1214. The parasitic types either fail to grow at all in the unsealed tube (*a*) seeded with abundant fresh growth, or else only one or several colonies or growth centers appear and attain normal dimensions. In some (*a*) tubes a very fine film may be detected with a hand lens but it does not acquire naked-eye dimensions. In the saprophytic types a distinct, even rich film may appear in 1 to 2 days on (*a*), also on (*b*). In the sealed tubes growth on (*a*) fails to distinguish the two types. In the tube receiving a loop of dilution (*b*) growth also appears in tubes of both types, but much later in the parasitic type. The distinctions become clearly marked in (*c*) and higher dilutions. As a working guide sealed tubes should be kept in the incubator about 15 days although growth appears only rarely after the 10th day. The continued vitality of the transferred bacteria when growth fails to appear after 10 or more days has been tested by unsealing and placing them in a jar containing 2 to 5 per cent CO_2. The originally unsealed tubes frequently failed to grow at this time, whereas those kept sealed the same length of time developed into rich films in the CO_2 jar, depending on the degree of dilution. The slight drying of the unsealed medium may have killed the bacilli on the agar surface within the period mentioned. In unsealed tubes containing a large seed and but few growth centers up to 10 days, numerous additional colonies may appear in the CO_2 atmosphere. In the sealed and unsealed tubes, multiplication, if it goes on at all, takes place at first in the condensation water. A slight cloud-like deposit usually precedes by a day or two a vigorous surface crop of colonies. In the unsealed tubes this cloud in the condensation water may appear and remain the only sign of multiplication. In tubes of bouillon a distinction between the parasitic and saprophytic type may be seen in the clouding of the dilutions used to inoculate the slanted agar. Dilutions (*b*) and (*c*)

are clouded from the start because of the large number of bacteria added. Dilutions (*d*) and (*e*) become clouded in the saprophytic but

TABLE I.

Growth in Dilutions on Agar.

(The figures stand for the day on which growth appeared.)

Case No.	Immediate source of strain.	Unsealed.		Sealed.				
		a	*b*	*a*	*b*	*c*	*d*	*e*
735	Guinea pig.	1	2	—	4	6	10+*	—
916	" "	—	2	—	—	2	2	—
1114	" "	6 (1 colony.)	0	0	9	0	0	—
1119	" "	2	2	—	3	4–6	8+	—
1128	" "	1	9+	—	2	2	7+	—
1150	" "	4 (1 colony.)	—	2	13+	0	0	—
1167	Fetus.	0	0	2	7	13+	—	—
1167	1st guinea pig.	0	—	3	0	0	—	—
1167	2nd " "	0	—	6	10–12	0	0	—
1168	Fetus.	5 (3 colonies.)	—	3	5–9	0	0	—
1175	"	13 (1 colony.)	—	2	8	0	0	—
1175	1st guinea pig.	4 (1 colony.)	—	4	5	∪	—	—
1175	2nd " "	0	—	2	7+	8	0	—
1176	Fetus.	1	—	1	2	4–5	5–6	—
1176	Guinea pig.	2	5	—	4	4	5	5
1186	" "	2+	6+	—	3–4	4	3	4–6
1187	Fetus.	2	2	2	3–5	2	2–3	6
1195	Guinea pig.	0	0	2	9	0	0	0
1202	Fetus.	11+	0	2–3	11	0	0	0
1205	Guinea pig.	7–8	—	3+	10–12	17	14	—
1207	Fetus.	1+	—	1	3	7	7	7
1211	"	2–5	—	1–2	5	5–6	7–8	—
1212	"	0	—	3–8	13–14	0	0	—
1214	"	4–5	0	1–2	6–9	6–9	8–9	—

+ signifies only one or a few colonies on the day indicated, followed later by richer growth.

not in the parasitic group. When placed in a CO₂ atmosphere the latter become clouded in 3 to 4 days.

The rate with which saprophytic characters are assumed in successive cultures will probably vary with a variety of conditions, such as rapid transfers, the quality of seed, sealing, refrigeration after growth has ceased, and others. Aside from these, certain inherent characters of the individual strain play a part. Although the test described for determining the CO_2 requirements readily separates cultures into two classes, these are only rough classifications and within each class individual differences are observable. To determine at what rate the changes favoring growth in open tubes takes place, a strain growing quite feebly in sealed tubes was chosen. Successive transfers were made by using dilutions equivalent to (b) of the scale and the tubes sealed. The first tube developed a film of growth in 10 days. The 7th transfer developed in 6 days. The 10th transfer, after a total period of 65 days, developed in 3 days; all still in sealed tubes. A series of unsealed tubes with cotton wool plugs dipped in paraffin was carried on parallel with the above. The material transferred from tube to tube was not diluted in order to insure one or more centers of growth. After $4\frac{1}{2}$ months, a tube inoculated from a loopful of condensation water into which the entire film had been washed, denominated (a) in the scheme, developed in 5 days. In the original culture (a) failed entirely to multiply in such tubes. At this date the unsealed series appeared as follows: (a) 5 days, (b) 5 days, (c) 9 days. In the sealed series, (b) was 5 days, (c) 5 days, (d) 8 days. This particular strain was thus fairly well saprophytized in 16 transfers lasting $4\frac{1}{2}$ months. The beginning of changes in the cultured strains is frequently indicated by several successive crops of colonies. A few colonies may appear early. Within 1 or 2 days a second, much larger crop of minute colonies appears. Rarely a third crop can be distinguished. These crops probably represent degrees of adaptation to the new conditions. The virulence of the freshly isolated strains whether in the parasitic or the saprophytic group was the same for guinea pigs. In other words, it was not possible to foretell from the lesions produced in what group the strain would fall.

Sources of the Saprophytic Types.—The strains of both parasitic and saprophytic types in Table I were isolated from animals comprising one large herd. Earlier single strains from four other herds all belonged to the normal or parasitic type. The evolution of the

saprophytic from the parasitic type remains to be determined. The writer has kept in mind the possibility that the saprophytic strains may have originated in cultures used as vaccines and that during several passages through cows they may have regained to a certain degree the original parasitic character, since they do not multiply as freely in open tubes as do vaccinal strains. The problem needs to be studied in a herd in which vaccination has not been practiced and which has not received accessions from vaccinated herds.

In the herd in question only one animal has been examined which gave birth to a premature calf 9 days after arrival from a distant state. The culture isolated was of the normal or parasitic type. In one heifer, the first pregnancy ended in abortion associated with a saprophytic type. The second also ended in abortion but a strain approaching the parasitic type was recovered. In the test, two crops of colonies appeared, suggesting a modification of the earlier saprophytic type towards the parasitic level. A. Harms[4] studied 13 strains of *B. abortus* with reference to the time required to bring them to multiply without association with *B. subtilis*. She found considerable variation, probably due in most instances to recovery of injected saprophytized strains.

In a study of abortions in several groups of heifers in the same large herd, it was evident that some one type was present in each group and passed from animal to animal.[5] In one group of 34 treated with killed cultures of *B. abortus*, only the normal type was recovered in 9 first or second pregnancies. From a second group of 9 treated with a saprophytized living culture, the normal type was recovered from two clinically normal pregnancies and the saprophytic type from the udder of a third cow a year later. A group of 13 heifers, controls to the preceding group, yielded the normal type from 6 animals. A year later one of the other seven yielded the saprophytic type. In a second control group of 10 heifers, the saprophytic type was recovered from 3. In a third control group of 14 heifers, the saprophytic type was found in 4. Thus in these two last groups the parasitic type was not found.

[4] Harms, A., *J. Bact.*, 1924, ix, 272.

[5] Smith, T., and Little, R. B., Studies in vaccinal immunity towards disease of the bovine placenta due to *Bacillus abortus* (infectious abortion), Monograph of The Rockefeller Institute for Medical Research, No. 19, New York, 1923, 87.

CONCLUSIONS.

Strains of *Bacillus abortus* freshly isolated vary in their CO_2 requirements. The origin and sources of the strains growing with less dependence on CO_2 (or sealing) may be in vaccinal strains or possibly in continued existence in the udder. The importance of these possibilities makes it desirable that all strains isolated should be subjected to some such test as is outlined in these pages.

FURTHER DATA ON THE EFFECT OF VACCINATION AGAINST BOVINE INFECTIOUS ABORTION.

By THEOBALD SMITH, M.D., AND RALPH B. LITTLE, V.M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

In a recent monograph[1] two experiments were described dealing with the effects of vaccination on the incidence of abortion in heifers associated with *Bacillus abortus*. The first experiment dealt exclusively with the effect of living cultures on the first and second pregnancies. In the second experiment one group of heifers was treated with cultures killed by heat. In the publication referred to only the first pregnancies were reported. There are now available data on the second pregnancies which shed some additional light on the value of vaccination.

The methods pursued were the same as those described in the monograph. All fetuses obtainable were cultured and at the same time guinea pigs inoculated with washings from uterine swabs. The agglutinins towards *Bacillus abortus* in the blood were measured by a suitably graded series of tests.

All animals were treated with vaccine before the first pregnancy only. In Group A all heifers received 4 doses of a heated culture, 2 before and 2 after the first service. Similarly the group treated with a living culture before the first pregnancy was not injected thereafter.

The outcome of first and second pregnancies is shown in Table I. It will be noted that between the two pregnancies some animals were disposed of as of reduced economic value.

Although Group A treated with dead cultures appeared to possess

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[1] Smith, T., and Little, R. B., Studies in vaccinal immunity towards disease of the bovine placenta due to *Bacillus abortus* (infectious abortion), Monograph of The Rockefeller Institute for Medical Research, No. 19, New York, 1923.

considerable resistance when compared with the controls in the outcome of the first pregnancy, it failed to hold its advantage in the

TABLE I.

No. of pregnancy.	No. of animals remaining available.	Full-term pregnancies.		Abortions.	Per cent aborted.	Per cent aborted and with placenta diseased.
		Normal.	With placenta diseased.			
Vaccinated Groups.						
Group A. (Heated vaccine.)						
First.........	34	25	4	5	14.7	26.4
Second......	30	23	2	5	16.2	23.3
Group B. (Living vaccine.)						
First.........	9	9	(3)*	0	0	(33.3)*
Second......	9	9	0	0	0	0
Control Groups.						
Group Bc.						
First........ ..	13	6	0	7	53.8	53.8
Second.....	11	8	2	1	9.9	27.3
Group C.						
First........ .	9	4	1	4	44.4	55.5
Second.....	4	1	2	1	25.0	75.0
Group D.						
First.........	14	9	1	4	28.5	35.7
Second......	9	6	2	(1)†	(11.1)†	22.2
Summary of Controls.						
First.........	36	19	2	15	41.6	47.2
Second.....	24	15	6	2	8.3	33.3

* Normal births with *B. abortus* in uterine washings.
† Vibrionic.

second. Thus the actual abortion rate compared with the controls was as 14.7 to 41.6 per cent in the first and as 16.2 to 8.3 per cent in

the second. When we include full-term pregnancies with diseased placenta, the difference is, however, still in favor of vaccination, 23.3 to 33.3 per cent.

Group B treated originally with one dose of living cultures is of interest in having maintained complete resistance in the second pregnancy. In the first all pregnancies terminated normally, but in 3 of the 9 animals the uterine swab produced the characteristic disease in guinea pigs, although the placentas were normal. In the second parturitions, also normal, all uterine swabs failed to infect guinea pigs. Although in one animal the placenta was retained, guinea pig tests of uterine swab and scrapings of chorion were negative for *B. abortus*. Sections of the placenta of this animal failed to show lesions and the cause of the retention remained undetermined. The

TABLE II.

Time.	Normal pregnancies.	Abortions.	Adherent placentas.	Per cent aborted.	Per cent aborted and with adherent placentas.
1924					
Jan..............	39	5	2	12.8	18.0
Feb.............	42	8	5	19.0	30.9
Mar............	37	6	6	16.2	32.4
Total........	118	19	13	16.1	27.1

animals of this group had been distributed among older cows to increase the opportunities for infection. These results can scarcely be regarded as of no significance, even in view of the small number of animals in the group.

The heifers in the control groups, of which only two-thirds remained in the herd, show a marked reduction in actual abortions but a relative increase in full-term pregnancies with diseased, adherent placenta.

During 3 winter months when most of the parturitions in the vaccinated and control groups occurred, the abortion rate among the cows making up the remainder of the herd is given in Table II. There were no special tests made to determine the nature of the disease in these animals. The table represents older and seasoned animals. The total figures approximate closely those of the group which received the heated cultures.

CONCLUSIONS.

The partial protection afforded by four injections of a heated culture of *Bacillus abortus* of normal virulence during the first pregnancy is in part lost in the second. The superiority of a single injection of a living culture of relatively low virulence is evident in both pregnancies. In the experiment described, the protection was complete.

A RAPID METHOD FOR THE ISOLATION OF BACILLUS ABORTUS FROM UTERINE EXUDATE AND DISEASED PLACENTA.

BY JOHN B. NELSON, PH.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

The diagnosis of contagious abortion in cattle not infrequently rests on the isolation of *Bacillus abortus* from infected material. The organisms may be present in such small numbers that repeated microscopic examination of films fails to reveal them. Uterine swabs and tissue from fetal membranes are usually contaminated with miscellaneous bacteria either from the utero-chorionic space or fecal matter or from both sources. The isolation of *Bacillus abortus* from such material is a matter attended with considerable difficulty. The preponderance of rapidly growing saprophytes together with the peculiar growth requirements of the microorganism itself make the use of simple plating methods for its recovery often very laborious and liable to failure and loss of material.

Animal inoculation has been resorted to as the most satisfactory method. Smith and Fabyan[1] showed, in 1912, that the injection of cultures of *Bacillus abortus* into the guinea pig gave rise to a disease of rare fatality, characterized chiefly by enlargement and congestion of the spleen together with foci of cellular proliferation. The splenic lesions began about the 3rd week, extended over a period of 10 to 20 weeks, and were followed by repair.[2] The disease could be transmitted from animal to animal by the injection of suspensions from a spleen in the acute stage. In 1918, Smillie[3] found that cultures could be obtained from the spleen 3 to 4 weeks after injection of the guinea

[1] Smith, T., and Fabyan, M., *Centr. Bakt., 1. Abt., Orig.*, 1912, lxi, 549.

[2] Fabyan, M., *J. Med. Research*, 1912, xxvi, 441.

[3] Smillie, E. W., *J. Exp. Med.*, 1918, xxviii, 585.

pig before definite gross lesions had appeared. The number of organisms in the spleen were found to be at their maximum between the 3rd and 4th week. After the 4th week a sharp decline in numbers was noticed.

These methods of isolation are reliable but time consuming, inasmuch as an interval of at least 3 weeks is necessary between injection of the guinea pig and cultivation from the spleen. An attempt to diminish the time interval before cultivation could be resorted to has led to the method to be described. The procedure is based on the observation made by Dr. Theobald Smith that the peritoneum of the guinea pig rapidly destroys most bacteria, excepting the spore stage, when injected in small numbers and it has been used in attempts to isolate certain bacteria in pathological material by. timing the exposure in the peritoneal cavity. *Bacillus abortus* is unaffected by the normal defensive factors operative in the peritoneal cavity which are accountable in part for the sterilizing action.

Procedure.

The method employed is as follows:

A suspension in bouillon is made from the suspected material. If it is a uterine swab, it is rotated vigorously until a noticeable cloudiness results. If placental tissue is available, it is thoroughly washed in running water, scrapings from the diseased chorion or cotyledons removed to a small crucible or convenient vessel and ground with sterile sand. Bouillon is added and after subsidence of coarse particles the supernatant liquid is employed. Approximately 1.5 to 2 cc. of the suspension is injected intraperitoneally into each of two or more guinea pigs. If a preliminary microscopic examination of the material shows streptococci in abundance, it is well to reduce the dose to 1 cc. Guinea pigs weighing around 350 gm. are to be preferred.

After 5 days one animal is chloroformed, the abdomen exposed and the muscles thoroughly seared with a hot spatula. With strict aseptic precautions a medial incision is made and several cc. of bouillon introduced into the peritoneal cavity with a blunt and smooth tipped pipette. The cavity is washed out by withdrawing and expelling the bouillon a number of times. Roughly 0.5 cc. of the washings is added to each of several agar slants.· The spleen is exposed, a bit of tissue torn off with sterile forceps and transferred to an agar slant. The cultures are placed in a closed jar in an atmosphere of 5 per cent carbon dioxide and incubated at 37°C. If *Bacillus abortus* is present growth either as isolated colonies or as a confluent film should occur in from 2 to 7 days. If reasonably strict aseptic

precautions are observed no contaminated tubes should be encountered. In case the cultures from the first animal remain sterile or are overgrown by bacteria other than *Bacillus abortus*, the second guinea pig should be killed and the procedure repeated. A third animal should be reserved and killed after 3 or 4 weeks.

Application of the Method.

The method described was tested with material from 12 cases of suspected bovine abortion. The results are presented below in tabular form. In addition, two cases from which *Bacillus abortus* was recovered are described in detail. Three and sometimes four guinea pigs were employed in order to ascertain roughly the length of time *Bacillus abortus* remained in the peritoneal cavity. The amount of growth on cultures from successive guinea pigs was simply estimated. Frequently isolated colonies were absent, growth occurring as a confluent film. Wherever possible the actual number of colonies is given.

Case 1176.—Uterine swab and a full-term fetus were received for examination. Autopsy of the fetus showed the presence of solid particles of meconium in the first and fourth stomachs. A pure culture of *B. abortus* was obtained from the lung, liver, first and fourth stomachs, spleen, and rectum. A culture from the kidney was without growth. Microscopic examination of films from the uterine swab showed microorganisms resembling *B. abortus*. 12 days later, the swab meanwhile being kept in the refrigerator, four guinea pigs were injected with 1.5 cc. portions of the swab suspension, three by the intraperitoneal route and one by the intrapleural route. Two guinea pigs were chloroformed after 5 days and cultures made from the washings of the peritoneal cavity in one case, of the pleural cavity in the other case. Spleen cultures were made from both animals. The turbid washings after subsidence showed many cells of which a small lymphocyte was the predominating type with smaller numbers of mononuclear and polynuclear leucocytes. There was evidence of phagocytosis in the two latter cell types. Cultures from the pleural washings showed in one case contamination, in others 35 and 6 colonies of *B. abortus*. The spleen culture showed a scant growth of *B. abortus*. Cultures from the peritoneal washings in one case showed contamination, in the others 2 and 4 colonies, respectively, while the spleen showed a scant growth. The third guinea pig was chloroformed 8 days after injection and cultures made from the peritoneal washings and from the spleen. The fluid was turbid and showed many lymphocytes with an occasional mononuclear and polynuclear leucocyte. 4 days later cultures from the washings showed 18, 10, and 12 colonies, respectively. The spleen gave a heavy confluent film. The fourth guinea pig was killed and cultured 14 days after injection. The washings showed a visible turbidity with cells in the same proportion as before. Cultures from the peritoneal

TABLE

Cultures from the Peritoneal Washings

Case No.	Nature of material.	Micro-scopic examina-tion of films.*	Days after injection.	Abdominal washings.†				
1175	Uterine swab.		5	6	—	50		
			11	1	—	2	—	10
			17	0	—	0	—	2
			74					
			107					
1176	" "	+	5	4	—	2	—Contaminated.	
			8	18	—	10	—	12
			14	1	—	2	—	0
1185	" "	—	5	Contaminated.—		0	—	0
			11	0	—	0	—	0
			21	Contaminated.—		0	—	0
			28	0	—	0	—	0
1186	" "	—	6	Scant.	—	Scant.	—	Scant.
			10	"	—	"	—	"
			21	0	—	0	—	12
1187	" "	—	6	8	—	5	—	7
			11	2	—	6	—	4
			21	0	—	0	—	0
1191	" "	—	5	0	—	0	—	0
			12	0	—	0	—	0
			19	0	—	0	—	0
			26	0	—	0	—	0
1195	" "	—	7	0	—	0	—	0
			14	1	—	0	—	0
			21	0	—	0	—	1
1202	" "	+	3	Moderate.	—	Moderate.	—	Moderate.
			12	Scant.	—	"	—	"
			19.	2	—	3	—	10

* + = *B. abortus;* − = *B. abortus* not found.
† The numbers represent colonies of *B. abortus.*

I.

and Spleen of Injected Guinea Pigs.

Spleen.†	Order of guinea pig examination.	Remarks.
30	1st	*B. abortus* isolated from fetal organs.
100	2nd	
80	3rd	
7	4th	
65	5th	
Scant, confluent.	1st	
Heavy, "	2nd	" " " " " "
175	3rd	
0	1st	Abortion with retained placenta, fetus lost, agglu-
0	2nd	tinins for *B. abortus* negative—probably vibrionic.
0	3rd	
0	4th	
Scant, confluent.	1st	
Heavy, "	2nd	
Moderate, "	3rd	
18	1st	Cultures from fetal organs positive for *B. abortus*.
40	2nd	
150	3rd	
0	1st	*Vibrio fetus* isolated from fetal organs.
0	2nd	
0	3rd	
0	4th	
0	1st	Cultures from fetal organs negative.
24	2nd	
100	3rd	
Moderate, confluent.	1st	*B. abortus* isolated from fetal organs.
Heavy, "	2nd	
" "	3rd	

TABLE I.

Case No.	Nature of material.	Micro-scopic examina-tion of films.*	Days after injection.	Abdominal washings.†				
1205	Uterine swab.		5	Scant.	—	Scant.	—	Scant.
			12	Contaminated.	—	Contaminated.	—	"
			19	0	—	0	—	"
1207	" "	—	5	Scant.	—	Scant.	—	10
			11	"	—	"	—	0
			18	0	—	0	—	Scant.
1211	Fetal membrane.	+	5	65	—	55	—	40
			13	Scant.	—	Scant.	—	0
			20	0	—	· 0	—	0
1214	Uterine swab.	+	5	Scant.	—	Scant.	—	Scant.
			12	"	—	"	—	"
			19	0	—	0	—	0

washings in one case gave no growth, in others showed 1 and 2 colonies, respectively. The spleen gave a pure growth of 175 colonies.

Case 1187.—Uterine swab and fetus were received for examination. Autopsy of the fetus showed the presence of solid particles of meconium in the fourth stomach. Pure cultures of *B. abortus* were obtained from the lung, first and fourth stomachs, and the rectum. Cultures from the spleen and liver were without growth. Microscopic examination of films from the uterine swab failed to show *B. abortus*. Three guinea pigs were each injected intraperitoneally with 1.5 cc. of a bouillon suspension made from the uterine swab. One guinea pig was chloroformed after 6 days and cultures made from the peritoneal washings and from the spleen. Cultures from the washings showed 8, 5, and 7 colonies, respectively, of *B. abortus;* the spleen showed 18 colonies. 11 days after injection the second guinea pig was killed and cultured. Cultures from the washings showed 2, 6, and 4 colonies, respectively, and from the spleen 40 colonies of *B. abortus.* 21 days after injection the third animal was killed and cultures made from the peritoneal washings, the liver, and the spleen. Cultures from the washings and the liver were without growth. The spleen culture showed 150 colonies of *B. abortus.*

DISCUSSION.

The injection of a mixed suspension of tissue fragments containing *Bacillus abortus* and other bacteria into the peritoneal cavity of the guinea pig is followed by a vigorous, differential sterilization whereby

—Concluded.

Spleen.†	Order of guinea pig examination.	Remarks.
Scant, confluent.	1st	Cultures from 2nd guinea pig, with one exception,
Contaminated.	2nd	showed *B. paratyphosus.*
Heavy, confluent.	3rd	
Moderate, "	1st	*B. abortus* isolated from fetal organs.
" "	2nd	
" "	3rd	
150	1st	
Moderate, confluent.	2nd	" " " " " "
" "	3rd	
Scant, "	1st	
65	2nd	" " " " " "
Heavy, confluent.	3rd	

after a few days and for a space of several weeks that microorganism is left in pure culture. After the 1st week, following injection, there is noted a gradual decrease in numbers. Reduction continues until sterility is reached, generally about the end of the 3rd week. During the same period of time there is a gradual increase in the number of microorganisms in the spleen. In no case, however, in the series studied were gross lesions in the spleen observable at autopsy. *Bacillus abortus* could be obtained in pure culture from the spleen as early as the 3rd day following injection and regularly on the 5th day. The cultures obtained from the spleen during this early period are probably due in part to the growth of organisms adhering to its surface. It is evident, however, that *Bacillus abortus* is carried very early into the circulation, and that growth is largely due to organisms actually in the splenic tissue. In one case, as noted in the case reports, the suspension was injected into the pleural cavity of a guinea pig and a pure culture of *Bacillus abortus* obtained from the spleen on the 5th day.

Cultures made directly from the blood gave irregular results. In one instance cultures made from the heart's blood 48 hours after injection were without growth. In another instance cultures made from the

heart's blood every 12 hours for 3 days after injection were all positive but with a scant growth. Cultures made from other organs, such as the liver, gave inconstant results. In one case the liver culture showed 2 colonies of *Bacillus abortus* while the spleen showed over 100. In two other cases the liver cultures were without growth while those from the spleen showed 150 and 25 colonies, respectively.

The factors accountable for the differential sterilization in the peritoneal cavity of the guinea pig were not studied. Undoubtedly many of the miscellaneous bacteria encountered are saprophytes and as such unable to adapt themselves to a parasitic mode of existence. Other bacteria, facultative parasites of a low order of virulence, must succumb to the normal defensive mechanism of the host. The injection is invariably attended by a cellular mobilization composed chiefly of small lymphocytes together with smaller numbers of mononuclear and polynuclear leucocytes. There is active phagocytosis, as evidenced by intracellular bacteria seen in stained preparations made from the peritoneal washings. It is to be anticipated that now and then streptococci or other pathogenic bacteria of sufficient virulence to overcome the normal defenses of the guinea pig may be present in the material employed for injection. Under these conditions a safe procedure would be to inject one or two guinea pigs subcutaneously. These usually survive even in the presence of streptococci or other pathogenic bacteria and may be cultured after the 3rd week.

SUMMARY.

A rapid method is described for the isolation of *Bacillus abortus* from the peritoneal cavity of the guinea pig following the injection of uterine exudate or placental tissue.

A STUDY OF BACILLUS ACIDOPHILUS FROM THE DIGESTIVE TRACT OF CALVES.

By MARION L. ORCUTT.

(*From the Department of Animal Pathology, The Rockefeller Institute for Medical Research, Princeton, N. J.*)

In investigations (Smith and Orcutt, 1925) carried on upon the early diseases of young calves, notably an early diarrhea known as scours the regular presence of certain Gram-positive bacilli and cocci in the normal digestive tract made it appear desirable to study these forms in detail. Two forms were manifestly predominant according to films from various levels of the digestive tract although other species, notably sporebearing anaerobes, were present in small numbers. The present article deals with the dominant Gram-positive bacillus, *Bacillus acidophilus;* the following paper with a second common form, designated as the enterococcus.

The literature contains only a few references to acidophilus bacilli from calves. Küthe (1915) made a study of bacteria in the intestinal tract using material from the stomach, duodenum, jejunum, ileum, cecum, colon, and rectum. He noted the appearance and reaction of the contents, made direct microscopic examination of stained smears, and cultured the material aerobically and anaerobically using plain agar plates, acetic acid bouillon, and deep layers of sugar agar. He always found a bacillus which he called *Bacillus acidophilus-polymorphus.* He noted the bacilli in the smears from the contents, usually of all levels, and he cultivated them in good numbers in the acid bouillon while the development in the non-acid cultures was more irregular and uncertain.

The present work was done with material from segments of the small intestine from the duodenum to the ileocecal valves and usually with material from the fourth stomach and sometimes from the cecum. The calves represent two groups, namely, 8 normal calves, from one to two days up to two to three months of age; and 14 calves having diarrhea, from one to eight days old. These calves had received a milk

diet. Among all animals of both groups Gram-positive rods appeared in varying numbers throughout the segments. Usually they were very numerous in the fourth stomach and the predominating type in that region. In the normal cases they were the predominating type throughout the small intestine, or they occurred in more or less equal numbers with Gram-positive cocci. In cases with scours a colon type of organism predominated in the lower segments (Smith and Orcutt, 1925).

In the beginning of the work the material was cultured on standard agar plus horse blood and only a few acidophilus colonies developed on this medium. The reason for the small numbers was probably due to the use of a too alkaline medium. The necessity of a certain degree of acidity for the cultivation of these forms was shown by the results obtained by varying the reaction of the medium, in 2 cases in which according to the films *Bacillus acidophilus* was present in large numbers.

These results indicated that the *B. acidophilus* types were living throughout the small intestine and that ordinary standard agar adjusted to a suitable acidity (pH 6.8) plus horse blood furnished a good medium for the isolation of this organism. Although pH 6.8 has proved a favorable H-ion concentration for an abundant growth, if the acidophilus bacilli were mixed with colon types the agar could be adjusted to pH 5.0, which is still favorable to *B. acidophilus* but harmful to *B. coli*.

The question arose as to whether the acidophilus bacilli actually multiplied in the intestine or were only carried down mechanically from the fourth stomach. To answer this three normal calves were examined carefully by plating measured amounts of material from the fourth stomach and the segments of the small intestine and counting the colonies which developed. All cases gave similar results, two of which are tabulated.

The figures show that in the segments just below the stomach *B. acidophilus* occurs in smaller numbers than in the fourth stomach. However, they also show that the acidophilus bacilli are present in large numbers throughout the small intestine and that in every case they exist in greater numbers in the lower segments than in the duodenum. These results suggest that *B. acidophilus* multiplies in the small intestine. The amount of increase varied in the different seg-

ments of the cases observed, probably depending on conditions existing in the different regions at different times. Also the multiplication in the intestine is not as abundant as in the fourth stomach although a certain amount does occur.

This group of organisms was first isolated and named by Moro (1900). All the earlier reports describe the strains as producing acid but no gas from carbohydrates. Torrey and Rahe (1915) described a gas-producing type which they named *B. acidophil-aerogenes*. They

TABLE I.*

Calf	Region	Occurrence (in films)	Cultivation (standard agar plus blood)
			pH 7.4–7.8
	Fourth stomach	++++	++++
	Duodenum	+++	+
	Second segment	+++	0
	Third segment	++	0
1065	Fourth segment	+++	+
	Fifth segment	+++	0
	Sixth segment	+++	0
	Ileum	++++	+
	Cecum	++++	+
			pH 6.8
	Fourth stomach	++++	++++
	Duodenum	+++	+++
	Second segment	+++	+++
1106	Third segment	+++	++++
	Fourth segment	+++	+++
	Ileum	++	+++

* ++++ indicates large numbers; +++, fair numbers; ++, moderate numbers; +, a few; 0, not cultured.

isolated this gas-producing type from the feces of man, sheep, and hens. They described two types of colonies appearing on glucose oleate agar plates, and also noted that in sugar bouillon the gas types were inclined to grow in particles adhering to the sides and bottom of the tube and tended to produce more acid than the non-gas types. The amount of gas produced varied with different strains and also from time to time with the same strain. The maximum gas production reported was 60 per cent and the minimum 2 per cent. They stated that the gas

was chiefly hydrogen and that the ratio of H to CO_2 was 4:1 or 6:1. Later Rahe (1918) presented a classification of aciduric bacteria. He named four groups: *B. bulgaricus*, *B. bifidus*, *B. acidophilus*, and *B. acidophil-aerogenes*. These were subdivided into classes according to the fermentation of various sugars. The acidophil-aerogenes type was was the gas-producing group and *B. bulgaricus* was separated from the others by its failure to ferment maltose.

Recently Cannon (1924) reported a biologic study of aciduric bacteria. He referred to the three main groups described in the literature, namely, *B. acidophilus* (Moro), *B. bifidus* (Tissier) and *B. acidophil-aerogenes* (Torrey and Rahe). Cannon worked with 64

TABLE II.

Calf	Region	Number of *B. acidophilus* per cubic centimeter
1106	Fourth stomach	1,330,000
	Duodenum	261,000
	Second segment	859,000
	Third segment	4,000,000
	Ileum	700,000
1112	Fourth stomach	7,820,000
	Duodenum	930,000
	Second segment	1,642,000
	Third segment	1,865,000
	Fourth segment	5,224,000
	Ileum	690,000

strains of *B. acidophilus* and 34 strains of *B. acidophil-aerogenes* isolated chiefly from sputum and feces of normal human adults. None of his strains were obtained from calves. He described the bacilli as facultative anaerobes but growing well under aerobic conditions. They did not liquefy gelatin. Some strains coagulated milk and some did not. In his study on the effect of the hydrogen-ion concentration he reported that a pH between 6.0 and 7.0 was most favorable but the majority of cultures grew on media adjusted from pH 4.8 to 7.6.

Küthe's paper (1915) is the only report describing acidophilus types from calves. The chief form which he found he called *B. acidophilus-polymorphus*. This organism he described as a Gram-positive rod

with rounded ends often occurring in long chains and sometimes in in single cells. These shorter single forms were sometimes slightly motile. On agar it formed dewdrop-like colonies which, when magnified, appeared like anthrax colonies. It also grew in a deep layer of glucose agar appearing in twenty-four hours as small dots, and when magnified the colonies showed smooth or slightly notched edges. In a stained preparation from an anaerobic colony the bacilli were thick and occurred singly and in pairs; sometimes there was a long thick rod with a short form above it like a head, also some comma forms and swollen rods. When transplanted back again under aerobic conditions the colonies were of the grayish dewdrop appearance and the bacilli grew again in chains, but the elements were thicker and more irregular than in the first aerobic culture. The alternate cultivation from anaerobic to aerobic conditions could be continued through a long series. Küthe considered *B. acidophilus-polymorphus* to be a single organism characterized by great pleomorphism and variation from aerobic to anaerobic growth. He further described this bacillus as coagulating milk and reddening litmus whey in twenty-four hours. It did not grow on gelatin and its development on serum was doubtful. It produced no indol and formed no gas. Küthe also mentioned two other rarer acidophilus types. These developed anaerobically and one type failed to grow in aerobic cultures.

The present report on calf acidophilus types includes studies of 17 strains recently isolated and 3 strains which were isolated from calves in 1917–1919 by Marian S. Taylor and have been kept in the collection of stock cultures here since that time. The three earlier strains and 12 of the recent strains belong to the class designated by Rahe, and later referred to by Cannon as *B. acidophilus;* and 5 strains, because of the production of gas, fall in the group called *B. acidophil-aerogenes*. In the *B. acidophilus* group 5 strains were isolated from the 4th stomach, one from the 4th segment, and one from the ileum, all from different calves; and 5 strains came from the duodenum of three different calves. In the *B. acidophil-aerogenes* group 2 strains came from the 4th stomach and 3 from the ileum.

All strains have certain morphological characters in common. They are all strongly Gram-positive rods, rather broad, and with blunt or square ends, showing considerable variation in length of

elements. Some rods are short, some long, and some occur in still longer filaments. The rods are single, paired, or in short or long chains, sometimes twisted and tangled. Certain differences occur among the strains. Some always appear as rather short forms usually in pairs, short chains, and groups, and often in parallel arrangement but with a certain variation in the length of the rods. Other strains show many long forms and develop long chains. These rods are usually straight and broad but the longer chains are often twisted or tangled. The gas-producing strains show the greatest variation. They are likely to show more curved rods and the chains are more tangled.

The growth of these strains has been observed in various media and the fermentation reactions of certain carbohydrates recorded. In the first place on the blood agar plates, which were used for the isolation of the cultures, all strains, both gas and non-gas types, showed a similar appearance. In twenty-four hours very tiny colonies with tiny greenish zones developed. Some colonies were only visible with the hand lens. In forty-eight hours the colonies were still small and showed small zones of greenish discoloration. The deep colonies ofter appeared irregular or jagged. The form of the surface colonies was seen better on the plain agar plates. From the different strains two or possibly three types of surface colony could be distinguished. All colonies were small delicate growths, usually not over 1 mm. in diameter. A few strains developed round, even, rather opaque colonies; other strains formed thin, delicate, irregular colonies; and the remaining strains developed small, delicate, more or less round colonies, but when examined with a hand lens threads were seen projecting from the edges. The round and irregular colonies occur in both gas and non-gas-producing types. In the same strain some colonies may be more jagged than others.

In standard bouillon (pH 6.8) the strains usually produce a small amount of fine sediment and a clouding made up of very fine suspended particles. In sugar bouillon the clouding is usually heavier and particles sometimes adhere to the sides of the tube. This is always the case with the gas strains. Certain strains do not cloud the bouillon but grow as a soft sediment made up of small particles. The strains which produced the sediment in bouillon also developed round col-

onies, but all strains forming round colonies did not grow as a sediment in bouillon.

All these acidophilus strains were grown in tubes of glucose agar 12 cm. deep. The cultures were inoculated when the agar was still soft after boiling. The medium was quickly solidified without shaking and incubated. The growth occurred throughout the depth of the agar. The colonies developed equally well from the top to the bottom of the tube, suggesting capacity for anaerobic growth.

An experiment with milk cultures was made by inoculating a series of milk tubes with different amounts of a bouillon culture and determining the pH values and the time when coagulation appeared. The amounts of culture used for inoculation were 0.01, 0.05, 0.1, 0.2, and 0.3 cc. The results showed that the tube inoculated with the largest amount of culture was the first and the one inoculated with the smallest amount the last to coagulate. The first sign of coagulation occurred in twenty-four hours in the tube receiving 0.3 cc. No coagulation was visible in the tube inoculated with 0.01 cc. until the 11th day. The coagulation always appeared first at the bottom of the tube. The original pH value of the milk was 6.7, and the H-ion concentration gradually increased. The greater the original inoculation the more rapidly the acidity increased. No visible coagulation occurred until a pH of about 5.8 was reached. A firm coagulum throughout the tube occurred at about pH 5.5 and the acidity might continue to increase gradually up to pH 4.8. The rate of coagulation depended upon the amount of culture inoculated. The greater the number of bacilli added the more rapid the coagulation; but regardless of the amount of the inoculation, no coagulation was visible until a rather definite pH value was reached, and the coagulation was not complete until a second definite H-ion concentration was attained. In other words, the time factor of coagulation varied with the amount of culture transferred, but the relation between the pH values and the degree of curdling was constant, the curdling always appearing at a rather definite H-ion concentration. On the other hand, the final pH value attained was also variable according to the amount of material inoculated. All tubes inoculated with amounts of 0.05 cc. and below reached a final pH of 5.4 to 5.3 after three weeks, whereas the tubes inoculated with 0.1 cc. or more reached within the same

period a final pH of 4.8. A similar test was carried out with additional strains to see if this difference in final H-ion concentration was general.

Seven strains were used and the amounts inoculated were 0.2 and 0.02 cc. of a bouillon culture. In every case the same conditions occurred as in the first experiment; namely, no visible coagulation until the pH value was about 5.8; the coagulation was not complete until the pH value was about 5.5; and the final H-ion concentration after three weeks gave a pH of 5.3 to 5.1 for the tubes inoculated with 0.02 cc., and a pH of 4.8 to 4.6 for the tubes inoculated with 0.2 cc.

All the cultures grew in gelatin at 37°C., and after a month no liquefaction occurred. The gelatin solidified as soon as it cooled.

Some experiments were made to determine the effect of the hydrogen-ion concentration on the growth of these cultures. The limiting H-ion concentrations were determined and also the range giving the best growth. The procedure used was to prepare a series of tubes of bouillon with a pH range from 8.0 to 4.4. Standard bouillon was used and N/20 NaOH added to obtain pH values above 7.6, and acetic acid added for the pH values below 7.6. Each tube contained the same amount of medium and was inoculated with the same amount of a forty-eight hour agar growth suspended in sterile distilled water. The growth was measured by plating and counting the colonies and observing the clouding of the bouillon. The medium used for plating was standard agar pH 6.8 plus horse blood. The colonies were counted after 72 hours incubation. This experiment was made with five strains: two gas-producing strains, 1071 and 1106 (fourth stomach); and three non-gas types, 1078, 1106 (ileum), and 133. Strain 1078 represented the cultures forming more or less even colonies and producing a clouding in bouillon; strain 1106 (ileum) represented the cultures forming round colonies and growing as a sediment in bouillon; and, finally, strain 133 was used as a representative of those which had been under cultivation since 1917. The results for the different pH values, indicated by the appearance of the bouillon and the colony counts, showed that *B. acidophilus* develops over a fairly wide range of H-ion concentrations. The recently isolated strains, including the gas and non-gas types, gave a similar range, but the older strain differed somewhat. In every case there was no growth at pH 8.0 and 4.4. Among the recently isolated strains there was little or no

growth at pH 7.7 to 7.8, which indicated that the limiting H-ion concentration on the alkaline side was pH 7.8 to 8.0. Also among the recently isolated strains there was always some growth at pH 4.6, and the gas strains showed a better development than the non-gas types at this H-ion concentration. There was usually a good growth at pH 4.8, but the maximum development occurred between pH 7.0 or 6.8 and pH 5.2. Strain 133 agreed with the other strains in its limiting H-ion concentration on the alkaline side but it had a higher pH value as the acid limit. This strain had been kept on standard agar of about pH 7.4, and this may account for the fact that it was less resistant to acid than the recently isolated strains. Its limiting H-ion concentration on the acid side was pH 5.2 to 5.0. The range for good growth was between pH 7.7 and 5.5. The optimum growth occurred from pH 7.4 to 6.0

The fermentation reactions of these acidophilus strains were tested on glucose, lactose, sucrose, maltose, and mannitol. Fermented bouillon (pH 6.8) plus 1 per cent of the sugar was used for these tests. One lot of bouillon was used throughout for each set of sugars. The pH values were obtained by the micro-colorimetric method described by Brown (1924). The per cent of acid produced was also obtained by the usual titration method. All strains produced a certain amount of acid from glucose, lactose, sucrose, and maltose, usually the greatest amount from glucose and the smallest amount from maltose. The gas-producing strains formed as a rule slightly more acid in glucose, lactose, and sucrose, and considerably more in maltose than the non-gas-producing types. Only one strain formed a small amount of acid from mannitol. Ten strains were tested in duplicate with 1 and 2 per cent of glucose. After two weeks the results in 7 cases showed practically the same pH values for the two tubes, and in the other 3 cases a slightly greater amount of acid occurred in the tubes containing the 2 per cent of sugar, the difference being the greatest in the gas-producing strains.

In regard to the relative amount of acidity produced the individual strains showed some differences. The titration results were rather more variable than the pH values. Kendall and Haner (1924) in a recent study of the metabolism of B. acidophilus refer to three morphologically different types. Two of their types fermented the same

sugars and the third type fermented two additional ones, sorbitol and mannitol. In regard to the relative amounts of acidity formed by each type in milk, and in gelatin containing glucose, lactose, and sucrose, they noted certain differences in the quantity of titratable acid produced and also in the rate of acid production. They mentioned that a resultant between the two factors, inherent acidogenesis and cultural adaptability, was the probable explanation of these differences.

Another reaction, which differs from time to time and varies for different cultures of the same strain, is gas production. The acidophil-aerogenes strains are quite variable in the amount of gas produced and in the time when gas appears. Thirty or more different records of strain 1071 in fermented bouillon plus 1 per cent glucose in fermentation tubes have been made. Just before these inoculations the strain was plated and a single colony picked in a series of five successive transfers. Each time the plates appeared pure. An agar slant was inoculated from a colony on the fifth plate and a transfer from this culture was used to inoculate the fermentation tubes. The following examples are mentioned to show the extent of the variations occurring in one strain. Two tubes of approximately the same size and filled to about the same level with one lot of bouillon were inoculated with equal amounts of material from the same culture. In one tube 4 per cent gas was formed in forty-eight hours and a total of 37 per cent appeared in six days; while in the second tube there was only a bubble of gas on the eleventh day and no further increase after fifteen days. A third tube was inoculated from the second and showed no gas after twelve days, although growth occurred as usual; and a fourth tube, inoculated from the third, again contained a considerable amount of gas. In another series, cultures were inoculated from the bulb, neck, and arm of a fermentation tube culture which contained gas. The results showed one culture inoculated from the neck which failed to produce gas, and a second tube inoculated from the same region which contained 34 per cent gas. All the tubes inoculated from the bulb and arm contained gas but in amounts varying from 10 to 31 per cent. Thus even with uniform conditions of the medium and the material for inoculation, the rate and amount of gas production still varied in different tubes. Also cultures containing little or no gas may give rise to cultures producing considerable gas and, *vice versa*, cultures

containing gas may give rise to others with little or no gas. Similar variations occurred in two other gas-producing strains tested in shorter series. Torrey and Rahe (1915) also reported variability in the amount of gas produced. However, the calf strains differ from the organisms studied by these workers in the composition of the gas. Torrey and Rahe state that the gas was chiefly hydrogen, but do not mention their method of measuring the gas. In the calf strains the gas, as it is formed in the fermentation tube, is almost totally absorbed by NaOH solution, indicating that it is chiefly CO_2.

An experiment was made by growing *B. acidophilus* and *B. coli* together in mixed culture. Fermented bouillon (pH 7.4) plus 1 per cent glucose was inoculated with a known number of *B. coli* and *B. acidophilus*. Pure cultures of each organism were made as controls. The growth and pH values were watched and it was found that *B. coli* grew more rapidly in the beginning, but when the pH value reached 5.2 to 5.0, which occurred after the first twenty-four hours, then *B. coli* ceased to multiply and *B. acidophilus* continued to increase. On the third day the control tube of *B. coli* alone showed the bacilli present in larger numbers than in the mixed culture. The counts gave 2228 M. *B. coli* per cc. in the control, in contrast to 32 M. per cc. in the mixed culture, while *B. acidophilus* was apparently still increasing in the latter. Thus *B. acidophilus* was able to outlive *B. coli* in the acid condition. On the other hand, if no fermentable substance is present, as indicated in a similar experiment of mixed cultures in plain bouillon, then *B. coli* will keep the pH value 7.0 to 7.5. This H-ion concentration is unfavorable for *B. acidophilus* but suitable for the growth of *B. coli*. In this experiment *B. coli* multiplied greatly and continued to live in large numbers for a week, keeping the ascendency over *B. acidophilus*. These results indicate that in mixed cultures *B. acidophilus* is able to check the growth of colon bacilli only when a fermentable substance is present. They are in agreement with Cannon's experiments with mixed cultures of *B. coli* and *B. acidophilus*. Similar experiments with mixed cultures of enterococci and colon bacilli, and also with mixed cultures of the three types, *B. acidophilus*, enterococci, and *B. coli*, are reported in a paper on enterococci from calves.

CONCLUSIONS.

1. *B. acidophilus* types are always present throughout the small intestine of young calves and are the predominating forms in the fourth stomach. In normal animals they are the predominating organism in the small intestine or they occur in more or less equal numbers with Gram-positive cocci.

2. *B. acidophilus* multiplies in the small intestine of normal calves but the degree of multiplication is greater in the fourth stomach than in the intestine.

3. Ordinary standard agar of pH 6.8 to 5.0 plus horse blood proved a suitable medium for the isolation of these organisms. Agar pH 6.8 is the most favorable for abundant growth but the more acid agar is better whenever colon organisms are numerous.

4. All the recently isolated calf strains showed certain characters in common, such as (*a*) similar optimum and limiting H-ion concentrations; (*b*) development of small greenish zones on blood agar; (*c*) growth throughout a layer of agar 12 cm. deep; (*d*) no liquefaction of gelatin; (*e*) coagulation of milk at definite pH values and final H-ion concentrations after three weeks, varying with the amount of material inoculated; (*f*) production of acid from glucose, lactose, sucrose, and maltose. The strains differed in the fermentation of mannitol; one strain produced a small amount of acid from this sugar and all the other strains failed to ferment it.

5. The calf strains may be divided into two groups according to gas production: (*a*) non-gas types, *B. acidophilus;* and (*b*) gas types, *B. acidophil-aerogenes.*

6. *Acidophil-aerogenes* strains are characterized by variation in the rate and amount of gas production, and the gas is always CO_2.

7. Experiments with *B. acidophilus* and *B. coli* in mixed culture, taken together with certain biological characters, indicate that *B. acidophilus* possesses a greater resistance to acid than *B. coli*.

8. The calf strains correspond in general in biological characters to *B. acidophilus* types isolated from man and other animals by earlier observers.

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A STUDY OF ENTEROCOCCI FROM THE DIGESTIVE TRACT OF CALVES.

By MARION L. ORCUTT.

(*From the Department of Animal Pathology, The Rockefeller Institute for Medical Research, Princeton, N. J.*)

A group of organisms described as Gram-positive cocci usually occurring in pairs and commonly found in the intestinal flora has been referred to under various names. Schmitz (1912) mentioned that the German literature contained references to *Micrococcus ovalis* (Escherich) and *Streptococcus lacticus* (Kruse) but seldom referred to enterococci. Thiercelin named this type of organism the enterococcus and hence in French publications this name is generally used.

Only brief references to the enterococcus from calves have been found. Küthe (1915) in his study of the bacteria of the intestinal tract of young calves states that cocci of various forms were frequently present in the intestines. These cocci were Gram-positive and grew well in acid bouillon. Tricoire (1917) in his review of the enterococcus states that enterococci occur in the intestines of cattle and some other animals. He described the organism as a micrococcus intermediate between streptococci and pneumococci, and appearing in animals and young cultures as a diplococcus, sometimes with a capsule or halo. The two cocci might be alike or different in size and form, and joined either in a straight line or at an angle. In older cultures the cocci were grouped in masses or chains and in very old cultures rod-like forms were found.

In the present study the same animals and material were used as decribed in the paper on *B. acidophilus* in calves (Orcutt, 1926); namely, the 4th stomach and small intestines, and sometimes the cecum, from a group of normal calves of 1 to 2 days up to 2 to 3 months of age, and similar material from sick calves 1 to 8 days old. Preparations from the intestinal contents stained by the Gram method showed Gram-positive cocci, usually in pairs. The cocci were sometimes

slightly elongated and the two elements of a pair sometimes varied in size and shape. A small lighter area might exist around the cocci with a line between the two elements of a pair, suggesting a capsule, but this was not always the case. Also the two cocci in the pair were arranged usually in a straight line but sometimes they were at an angle.

These cocci were present in considerable numbers in the films from practically all intestinal levels of normal calves. They also existed in varying numbers in the intestinal contents of calves suffering from diarrhea. In some cases rather large numbers, and in other cases only a few cocci were seen. The distribution of the cocci as indicated from the films showed that as a rule they were more abundant in the small intestine than in the 4th stomach although a few cases showed the cocci about equally numerous in both regions.

The development of the cocci on plates inoculated from the intestinal contents was variable and sometimes the growth did not correspond to the numbers seen in the films. Also, occasionally from a certain segment the cocci developed in almost pure culture. This indicated that multiplication could occur in the intestine, and the region where abundant growth took place probably depended upon the presence of substances which were favorable to the growth of the cocci.

These Gram-positive diplococci seen in the intestinal contents of the calves have been considered as enterococci. They form one of the groups of organisms always present in the intestinal tract of young calves and their existence there is probably a normal condition. They seem to be only saprophytic organisms, but according to a review by Tricoire they are able at times to produce disease. Tricoire (1917) stated that the enterococcus was a saprophyte of man and animals but that it might become pathogenic. He mentioned that it existed normally in the intestinal tract and was one of the fundamental elements in the intestinal flora together with *B. coli* and *B. bifidus*. He listed various reported cases of enterococcus infection, including gastro-enteritis, hepatic infections, appendicitis, peritonitis, intestinal intoxications, lung abscess, rheumatism, and also cases of tuberculosis, typhoid and paratyphoid fever with which the enterococcus was associated. Furthermore the organism has been reported in wound

infections. Thus numerous cases are on record in which infection is ascribed to an organism designated as enterococcus. In the intestines of the calves used in this study the enterococci occurred in considerable numbers, but there was no disturbance referred to their presence.

Tricoire (1917) described the enterococcus as a facultative aerobe growing at room temperature but with an optimum temperature for growth at 37°C. It developed readily in bouillon and peptone solution, and after 24 to 48 hours it produced a sediment, rising in a spiral without breaking up when shaken. It coagulated milk, did not liquefy gelatin, formed no indol, and generally did not ferment sugars.

Dible (1921) considered the enterococcus of Thiercelin, the *Micrococcus ovalis* of Escherich, and the *Streptococcus fecalis* of Andrewes and Horder as probably belonging to the same group of organisms. He studied a large number of strains isolated from feces and concluded that the enterococci were a rather well defined group which could be differentiated from the streptococci by resistance to heat. He designated a central type and three variants. He described the enterococcus as a lanceolate diplococcus growing in bouillon, sometimes in short chains. Occasionally a slight halo was seen around the cocci, but no capsule was demonstrated. It grew slightly in media alkaline to phenolphthalein and gave no growth in media acid to Congo red. In bouillon it formed a uniform turbidity. It coagulated milk and did not liquefy gelatin. It was insoluble in bile and had no action on red blood corpuscles, producing neither hemolysis nor methemoglobin. The main type fermented lactose, salicin, sucrose, and mannitol. One variant failed to ferment sucrose, a second failed to ferment mannitol, and a third failed to ferment both sucrose and mannitol. None fermented raffinose and inulin. Besides these four groups he also mentioned an occasional variant capable of fermenting raffinose. He stated that enterococci occurred constantly in stools but were not particularly associated with diarrhea. In a few instances he found the organism in saliva.

Kendall and Haner (1924) studied the metabolism of *Micrococcus ovalis*. They state that Escherich isolated and described this organism in 1886, and that in 1899 Thiercelin rediscovered it and named it enterococcus. Kendall and Haner isolated enterococci frequently from the duodenum of adults. Their cultures represented the four

types of Dible and also three additional variants. They considered the fermentation of salicin of importance for identification. They found certain strains which fermented inulin but differed from pneumococci by being insoluble in bile. They considered that the enterococci were normal intestinal microbes of the lactic acid forming type.

The present study comprises twelve cultures from calves. They showed certain differences among themselves. One form agreed with the type organism of Dible. There were three variants, one like Dible's variant I, a second like Kendall and Haner's variant IV, and the third and largest group like variant V of Kendall and Haner.

Their appearance in films from intestinal contents has already been mentioned. In 24 or 48-hour agar cultures all strains gave a similar picture. The cocci were mostly in pairs, scattered or grouped together, and some short chains were also seen. The elements were round or slightly elongated and rather irregular, frequently varying in size. An examination of preparations made with India ink showed no distinct capsules. In plain bouillon the individual strains showed certain differences. In some cultures the cocci were chiefly in pairs and varied in shape, being round, slightly elongated, or irregular. Other strains had a similar form but the cocci were generally smaller, and a third group showed a greater proportion in short chains and the elements more elongated. The India ink preparations from the plain bouillon cultures also showed no definite capsules. In sugar bouillon Strain 156 gave a viscid growth, and when examined with India ink large distinct capsules were visible. None of the other strains gave this type of growth in sugar bouillon nor did they show definite capsules. In regard to capsule formation, Schmitz (1912) states that the occurrence of capsules was not constant but sometimes they were present and under other conditions absent. Dible, and also Kendall and Haner mention that capsules were usually not demonstrable.

Various cultural reactions of these calf strains have been noted. In plain standard bouillon all the strains produced clouding and seven formed a viscid sediment rising in a spiral when the tube was shaken, and thus corresponding to Tricoire's bouillon growth. The other five strains formed a small amount of fine sediment, mixing easily when shaken. All strains coagulated milk. They all grew well in gelatin at

room temperature and caused no liquefaction. The growth on potato was slight. On horse blood agar plates the strains produced deep colonies with small greenish zones and round smooth grayish surface colonies also with small zones of greenish discoloration. These differ from Dible's strains which he stated had no action on red blood corpuscles, but Dible's method for testing the action of the culture on blood was different, which may account for his result.

The fermentation capacity was tested on glucose, lactose, maltose, salicin, raffinose, sucrose, mannitol, and inulin. The result divided the strains into four groups. All strains fermented glucose, lactose, maltose, and salicin. Two cultures failed to ferment sucrose, and these same two and two others did not ferment raffinose but all four gave a positive reaction with mannitol. Seven other strains gave a negative result with mannitol and a positive reaction with raffinose. One strain fermented all the sugars including inulin. As already mentioned, the four groups agree with the type organism and variants I, IV, and V of Kendall and Haner. The majority of the calf strains correspond to their variant V. These groups with the pH values for the calf strains have been tabulated.

These results indicate that the calf enterococci corresponds with certain types of the organism isolated from other sources. None of the calf strains in this collection agreed with variants II and III of Dible, or these same types plus variant VI of Kendall and Haner. The pH values showed a fairly uniform amount of acid produced by the different strains in each sugar whenever fermentation occurred, except Strains 1075, 1082, and 1094, which formed less acid from raffinose than the other cultures which fermented this sugar. Strain 1106 agreed with the inulin-fermenting type of enterococci and differed from pneumococci in being insoluble in bile. All the other strains of enterococci were also insoluble in bile.

In experiments on heat resistance 24-hour bouillon cultures in amounts of 2 cc. were heated in corked tubes in a water bath at 60°C. for 30, 15, and 5 minutes. One cc. of the heated culture was plated and one cc. added to fresh bouillon. After the cultures had been heated for 30 minutes, six developed clouding in bouillon and showed plates filled with colonies. Another strain resisted heating at 60°C. for 15 minutes, and two others showed a few resistant organisms

after 15 minutes exposure in one experiment and none in another; while three strains were consistently sensitive to heat at 60°C. for 15 minutes but withstood 60°C. for 5 minutes. It was noted that the seven strains forming a ropy sediment in bouillon were the most heat resistant. Dible considered heat resistance a character differentiating

TABLE I.

Fermentation Reactions of Calf Strains of Enterococci Grouped According to the Types of Kendal and Haner.

Groups		Fermentation reaction with pH values for calf strains							
Kendall and Haner	Calf strains	Glucose	Maltose	Lactose	Salicin	Sucrose	Man-nitol	Raf-finose	Inulin
Type culture				+	+	+	+	• —	—
	1070c	4.8	5.0	5.0	5.0	4.9	5.3	7.6	7.6
	156	4.8	4.9	5.0	5.0	4.8	5.3	7.6	7.7
Var. I				+	+	—	+	—	—
	193	4.9	4.9	5.1	5.0	7.6	5.4	7.5	7.5
	945	4.8	5.0	5.0	5.0	7.6	5.4	7.6	7.6
Var. II				+	+	+	—	—	—
Var. III				+	+	—	—	—	—
Var. IV				+	+	+	+	+	+
	1106	4.8	4.8	5.0	5.0	.4.8	5.2	5.0	4.8
Var. V				+	+	+	—	+	—
	1070 (2nd)	4.8	4.9	5.0	5.0	4.9	7.6	4.8	7.5
	1072	4.8	5.0	5.0	4.9	4.9	7.6	4.9	7.5
	1074	4.8	4.9	5.0	4.9	4.8	7.6	4.8	7.5
	1121	4.9	4.9	5.0	4.9	5.0	7.6	4.8	7.5
	1075	4.8	5.0	5.2	5.1	4.9	7.7	5.8	7.6
	1082	4.8	5.0	5.1	5.0	4.8	7.7	5.5	7.6
	1094	4.9	5.0	5.1	5.0	4.9	7.7	5 7	7.6
Var. VI				+	+	+	—	+	±

enterococci from streptococci. He stated that all enterococci in broth cultures survived heat at 60°C. for 15 minutes while streptococci were killed in 5 minutes and that he found only one exception to this rule. According to him, the property of heat resistance was due to the presence of some specially resistant cells since most of those present in the culture were killed.

The limiting and optimum H-ion concentrations were determined by the same method as that described in the paper on *B. acidophilus* except that ordinary standard agar was used for plating instead of agar adjusted to pH 6.8. Three strains were used, 1082, 1106, and 193. The growth after 24 hours changed the initial pH toward the acid side, probably because traces of muscle sugar were present. The organisms were able to grow in an alkaline medium of pH 9.0 and certain strains at a slightly higher pH. No growth occurred in bouillon with a pH value of 10.0. The optimum for all the strains tested was between pH 8.0 and pH 6.0 The acid limit varied with the individual strains. Two cultures multiplied at pH 5.0 but gave no growth at pH 4.8, and one strain failed to grow in bouillon of pH 5.5.

Three sets of mixed culture experiments were made in which the enterococci were grown with *B. coli*, with *B. acidophilus*, and finally with both of these organisms. Plain and glucose bouillon were inoculated with the mixtures and a pure culture of each reserved as a control. The strain of *B. coli* used was one isolated within the year from a case of scours. Colony counts were made to determine the number of organisms inoculated and the subsequent growth occurring in the tubes. In the first experiment with a mixture of enterococci and *B. coli*, the results showed that in the glucose bouillon both organisms grew abundantly in the first 24 hours. The pH value reached 5.0 on the 2nd day. From the 3rd day on the enterococcus kept the ascendency over *B. coli*. In the plain bouillon tube the two strains multiplied together to about the same degree and at the end of a week both were living in large numbers. The pH at this time was 7.8. Altogether the results indicated that the enterococcus is more resistant to acid than *B. coli*.

Experiments in which enterococci were mixed with *B. acidophilus* were carried out in plain and glucose bouillon of three different pH values,—namely, pH 7.4, 6.8 and 5.5. The mixed growth was controlled by pure cultures of each organism. The development was determined by noting the clouding of the bouillon, examining stained preparations microscopically, and making colony counts. The results showed that in bouillon of pH 7.4 and 6.8 the enterococci always grew very abundantly in the beginning, clouding the tubes in 4 to 5 hours, while *B. acidophilus* showed no visible growth at this time. After 24

hours the acidophilus control tubes were well clouded, indicating that *B. acidophilus* grows well in this medium if it is given time to develop without interference. The mixed culture tubes at this time contained chiefly enterococci. The results were similar in both plain and glucose bouillon. These pH values of 7.4 and 6.8 were within the range of optimum growth for enterococci which gave them the advantage. On the other hand, *B. acidophilus* is generally more resistant to acid than enterococci. In the tubes containing bouillon of pH 5.5 the enterococcus growth was delayed. This H-ion concentration is beyond its range of optimum growth but within that for *B. acidophilus*. In this series of tubes no visible clouding occurred within 4 to 5 hours, but after 24 hours all the cultures were clouded. The mixed culture showed acidophilus bacilli present in numbers equal to ·or slightly above those of the enterococci. This is in contrast to the development in tubes where the enterococcus multiplication was not delayed. However, after 48 hours the plain bouillon tubes in the pH 5.5 series showed a continued increase of enterococci resulting in their predominance in the mixed culture; while in the glucose bouillon mixed culture of this series according to stained preparations the enterococci did not predominate but were held in check, probably by the increased acidity. Altogether the results indicate that the enterococcus can prevent the growth of *B. acidophilus* by means of its more rapid multiplication, and that the acid reaction produced in the glucose tube has little effect on the development whenever the early rapid growth of the enterococcus can take place, for the multiplication of *B. acidophilus* was interfered with in both the glucose and plain bouillon tubes of an original pH of 7.4 or 6.8. However, the acid formed in these tubes tends in the end to favor *B. acidophilus* against enterococci, since acidophilus bacilli are slightly more resistant to acid, and in a few cases *B. acidophilus* has been found persisting in such cultures after 3 or 4 days. On the other hand, if the original acidity is beyond the range of optimum growth for the enterococci and within that for *B. acidophilus*, then the enterococcus growth is delayed and *B. acidophilus* has a chance to multiply. Thus two of the factors influencing the growth and final outcome of a mixture of enterococci and *B. acidophilus* are (1) that of the rapid growth of the enterococci, which is favored or interfered with by the original pH

value of the medium, but which in itself is harmful to the development of *B. acidophilus;* and (2) the acidity produced in the glucose bouillon, which in the end tends to favor *B. acidophilus* against enterococci.

The third type of mixed culture experiment, in which enterococci were mixed with both *B. coli* and *B. acidophilus,* agreed in its results with the other two groups of experiments. In this case, in the glucose bouillon both enterococci and *B. coli* multiplied greatly in the beginning. The pH reached 5.2 in 24 hours and 5.0 on the 2nd day. *B. coli* was the first organism to disappear and it was survived by the enterococci and *B. acidophilus.* The growth of *B. acidophilus* was checked in the beginning, for it was not recognized in the plate cultures during the first 4 days, but on the 8th day it was growing in the glucose bouillon culture. In the plain bouillon tube *B. acidophilus* was not recognized in plate cultures after the first 24 hours, but *B. coli* and the enterococci grew well together and at the end of a week both were living in large numbers. The pH of this culture ranged from 7.1 to 7.7. All the mixed culture experiments bring out the effect of the H-ion concentration in relation to the action of these strains against each other. They show that both enterococci and *B. acidophilus* are antagonistic to *B. coli* by their greater resistance to acid; but enterococci are antagonistic to *B. acidophilus* because of their multiplication in greater numbers, and the acid reaction produced by the two strains in mixed culture in glucose bouillon tends in the end to favor *B. acidophilus* against enterococci.

<div align="center">CONCLUSIONS.</div>

1. Enterococci were found to exist in the digestive tract of normal calves. They were also present in varying numbers in calves suffering from diarrhea, or scours. They sometimes developed in practically pure culture at certain levels.

2. The enterococcus organisms from calves do not represent a homogeneous group but are separated into four divisions according to fermentation reactions. These groups agree with the type form and certain variants described by Dible and by Kendall and Haner.

3. All the calf strains examined agreed in coagulating milk, leaving gelatin intact, producing green zones on blood agar plates, fermenting glucose, lactose, maltose, and salicin, and maintaining the same range

of H-ion concentration for optimum growth.   They differed in the fermentation of mannitol, sucrose, raffinose, and inulin; also in capsule formation, in the character of sediment in bouillon, in the degree of resistance to heat, and in the limiting H-ion concentration.

4. Enterococci growing in mixed cultures with *B. coli* are able to survive *B. coli* whenever acid conditions are produced.   Enterococci are also harmful to *B. acidophilus* when they are grown together in mixed cultures, unless the early development of the enterococcus is delayed.   In this case the injurious effect is not due to acid but rather to the rapid multiplication of the enterococci.   Whenever acidophilus bacilli survive in such a mixed culture they are found later in the tubes containing acid; thus the acidity tends in the end to favor *B. acidophilus* against enterococci.

5. The enterococcus differs from *B. acidophilus* in its ability to grow in a more alkaline medium, and also in being slightly less resistant to acid, although it is more resistant than *B. coli*.

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